

**The Roles of *Candida albicans*
Gpm1p and Tef1p in Immune Evasion and
Tissue Invasion of the Human Host**

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ABBREVIATIONS

Ab	antibody
AP	alternative pathway of the complement system
BSA	bovine serum albumin
C3aR	C3a receptor
C4BP	C4b binding protein
C5aR	C5a receptor
CFHR1	Factor H-related Protein 1
CP	classical pathway of the complement system
CR	Complement Receptor
CRlg	Complement Receptor of the Ig superfamily
DAF	Decay Accelerating Factor
DAPI	4'6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate buffered saline
ECM	extracellular matrix
Efb	extracellular fibrinogen-binding protein of <i>S. aureus</i>
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FHL-1	Factor H-like Protein 1
Gpd2	glycerol-3-phosphate dehydrogenase 2
Gpm1p	phosphoglycerate mutase
h	hour
HaCaT	human adult low calcium high temperature, keratinocytes
Hgt1p	high-affinity glucose transporter 1
hiNHS	heat-inactivated normal human serum
HRP	horseradish peroxidase
HSA	human serum albumin
HUVEC	human umbilical vein endothelial cell
iC3b	inactive C3b
iC4b	inactive C4b
IgG	immunoglobulin G
IPTG	isopropyl- β -D-thiogalactopyranoside
LB	Luria Broth
LPS	lipopolysaccharide
LSM	confocal laser scanning microscopy
MBL	mannose-binding lectin pathway
MCP	Membrane Cofactor Protein
MPO	myeloperoxidase
NHS-EDTA	normal human serum with 10 mM ethylenediaminetetraacetic acid

OD	optical density
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
Plg	plasminogen
Pra1	pH-regulated antigen protein 1
RGD	Arg-Gly-Asp binding domain of vitronectin
ROS	reactive oxygen species
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SG	Saboraud glycerol
TCC	terminal complement complex
Tef1p	translation elongation factor 1-alpha
tPa	tissue-type plasminogen activator
YPD	yeast peptone dextrose
YPGE	yeast peptone glycerol alcohol

SUMMARY

Human-microbial pathogen interaction is a constant battle between the two players whose aim is to overpower each other. During this interaction, a balance is maintained between the pathogen and the human host. However, opportunistic microbial pathogens start to overcome the human host when its physical and immune defenses get compromised. *C. albicans* is an opportunistic fungus which persists and causes muco-cutaneous and systemic infections in humans. *C. albicans* binds complement regulatory proteins Factor H, FHL-1, C4BP, and plasminogen which helps the fungus to inhibit further production of the opsonins C3b and iC3b, thereby evading the innate immune system. In addition, *C. albicans* binds to epithelial and endothelial cells to colonize and invade human tissues. Several secreted and cell surface proteins of *C. albicans* interact with complement proteins and adhere to host cells. However, homozygous deletions of the genes expressing the identified proteins do not totally abolish the interactions of *C. albicans* with the corresponding ligands, indicating that there are other *C. albicans* proteins which mediate the interactions. Moreover, mechanisms of the mentioned interactions are not fully understood. It is therefore the aim of this study to identify the roles of surface proteins of *C. albicans* and elucidate the mechanisms of their interaction with the cells and components of the human immune system.

In this dissertation, two surface proteins of *C. albicans* are characterized with regards to their roles in the immune evasion and tissue invasion of the human host. Specifically, *C. albicans* phosphoglycerate mutase (Gpm1p) binds the host glycoprotein vitronectin which mediates the adherence of the fungus to endothelial cells and keratinocytes as demonstrated by adherence to HUVEC and HaCaT, respectively. *gpm1* null mutant fails to adhere to endothelial cells whereas the Gpm1p-coated latex beads have increased adherence to endothelial cells as compared to BSA-coated latex beads used as control. These results indicate that Gpm1p mediates the adherence of *C. albicans* to endothelial cells. Furthermore, vitronectin is detected on the surface of HUVEC and HaCaT. Gpm1p colocalizes with the surface vitronectin of human cells illustrating that *C. albicans* Gpm1p adheres to human cells using vitronectin as ligand. A detailed investigation on the Gpm1p-vitronectin interaction revealed that Gpm1p binds vitronectin through its heparin binding sites and binding of Gpm1p to vitronectin is slightly affected by ionic conditions.

The translation elongation factor-1 alpha (Tef1p), another cell surface protein of *C. albicans*, is demonstrated in this dissertation to mediate *C. albicans* interaction with human complement proteins. Tef1p binds to complement regulatory proteins Factor H and C4BP; and to C3 fragments C3b, C3d, C3dg and iC3b. Tef1p binds the plasma protein plasminogen. The Tef1p-bound

plasminogen is activated to plasmin in the presence of the tissue-type plasminogen activator and maintains degradation activity on the synthetic substrate S-2251, extracellular matrix proteins fibrinogen and vitronectin, and the opsonin C3b. Thus, Tef1p binds to plasminogen to degrade ECM proteins for tissue invasion and to degrade the opsonin C3b to avoid recognition by phagocytic cells. Moreover, it is demonstrated in this dissertation that the *TEF1* gene is not essential in *C. albicans* as the *tef1* null mutant has the same growth rate as the wild type in normal culture conditions. *tef1* null mutant also grows normally as the wild type in the presence of osmotic and oxidative stressors, and cell-wall perturbing agents. These results are attributed to the presence of *TEF2* gene which also encodes for the Tef1p protein. However, the *tef1* null mutant is sensitive to the antimycotic drug hygromycin which directly inhibits the protein synthesis in *C. albicans*. *tef1* null mutant also binds less C3d and plasminogen as compared to the wild type. Thus, deletion of *TEF1* affects the binding properties of *C. albicans* to human complement proteins. Furthermore, Tef1p is involved in *C. albicans*-neutrophil interaction as the recombinant Tef1p binds to neutrophils and *tef1* null mutant fails to elicit reactive oxygen species (ROS) production in neutrophils.

Taken together, this dissertation demonstrates the importance of cell surface-associated proteins of *C. albicans* in the virulence of the pathogen, may it be for evasion of the human immune system or adherence to host tissues. Elucidating the mechanisms on how *C. albicans* uses its surface proteins can give light to possible strategies on how to combat this menacing fungal pathogen which could eventually lead to improvement of the quality of human life.

ZUSAMMENFASSUNG

Die Interaktion zwischen Mensch und Krankheitserreger ist ein immerwährender gegenseitiger Kampf mit dem Ziel, den jeweils anderen zu überwinden. Diese Interaktion resultiert in einem relativen Gleichgewicht zwischen Krankheitserreger und Wirt. Trotzdem sind opportunistische Krankheitserreger in der Lage, den Wirt zu besiegen, wenn dessen physikalische und Immunsystem-vermittelte Abwehr beeinträchtigt ist. *C. albicans* ist solch ein opportunistischer pilzlicher Krankheitserreger, welcher im Menschen persistiert und Schleimhaut- und systemische Infektionen auslösen kann. *C. albicans* bindet die Komplement-regulatorischen Proteine Faktor H, FHL-1, C4BP und Plasminogen. Diesen ist gemein, dass sie die Bildung der Opsonine C3b und iC3b inhibieren und somit eine Möglichkeit für den Pilz darstellen, die Mechanismen des angeborenen Immunsystem zu unterwandern. Außerdem bindet *C. albicans* an Proteine der Extrazellulären Matrix (ECM), welches die Kolonisierung und Invasion des menschlichen Wirtsgewebes erleichtert.

Eine Vielzahl sekretierter und Zellwand-gebundener Proteine aus *C. albicans* wurden identifiziert, die an ECM-Bindung und Interaktion mit Komplement-regulatorischen Proteinen teilnehmen. Durch Studien mit homozygoter Deletion der Gene, die für die entsprechenden Proteine kodieren, konnte gezeigt werden, dass dadurch die Fähigkeit von *C. albicans* an o.g. Liganden zu binden, nicht vollständig aufgehoben wird. Dies deutet darauf hin, dass *C. albicans* über weitere Proteine verfügen muss, die ebenfalls eine Interaktion vermitteln können. Zudem sind die genauen Interaktionsmechanismen für Bindung der genannten Liganden noch nicht vollständig verstanden. Daher ist das Ziel dieser Arbeit zum einen die Identifikation und Funktion weiterer Oberflächenproteine von *C. albicans* sowie zweitens die Aufklärung ihrer Interaktionsmechanismen mit Zellen des Menschen sowie den Proteinen des Immunsystems.

In der vorliegenden Dissertation werden zwei Oberflächenproteine von *C. albicans* im Hinblick auf ihre Rolle bei der Immunevasion und der Gewebsinvasion im Detail untersucht. Die Phosphoglyceratemutase (Gpm1p) aus *C. albicans* bindet das Wirtszell-Glykoprotein Vitronectin, was die Adhärenz des Pilzes an Endothelzellen und Keratinozyten vermittelt, beispielhaft gezeigt durch Adhärenz an HUVEC bzw. HaCaT. *gpm1* Deletionsmutanten sind nicht in der Lage, an Endothelzellen zu adhären, wohingegen Latex-Beads, welche mit Gpm1p beschichtet wurden, eine Bindung an Endothelzellen zeigen, was durch Verwendung von BSA-beschichteten Latex-Beads als Negativkontrolle nicht der Fall war. Dies deutet darauf hin, dass Gpm1p in der Tat die Adhärenz von *C. albicans* an Endothelzellen vermitteln kann. Außerdem konnte Vitronectin direkt auf der Oberfläche von HUVEC und HaCaT nachgewiesen werden.

Folgeexperimente zeigen eine Kolo-kalisation von Gpm1p mit Vitronectin auf den humanen Zellen, was dafür spricht, dass *C. albicans* Gpm1p Vitronectin als Ligand für die Adhärenz verwendet. Detaillierte Studien der Interaktion zwischen Gpm1p und Vitronectin zeigen weiterhin, dass Gpm1p Vitronectin über die Heparin-Bindestellen bindet und die Stärke der Bindung durch ionische Bedingungen variierbar ist.

Der Translations-Elongationsfaktor-1 α (Tef1p) stellt ein weiteres Zelloberflächenprotein von *C. albicans* dar. In dieser Arbeit konnte gezeigt werden, dass Tef1p die Interaktion von *C. albicans* mit Proteinen des menschlichen Komplementsystems vermittelt. Tef1p bindet die Komplement-regulatorischen Proteine Faktor H und C4BP, sowie die C3 Spaltprodukte C3b, C3d, C3dg und iC3b. Außerdem bindet Tef1p an das Plasmaprotein Plasminogen. Gebunden an Tef1p, kann dieses in Anwesenheit des Gewebsspezifischen Plasminogenaktivators zu Plasmin aktiviert werden und behält seine Aktivität der Degradierung bei. Letztere wurde getestet durch Zugabe an verschiedenen Substraten, u.a. das synthetische Substrat S-2251, sowie an den ECM-Proteinen Fibrinogen und Vitronectin, bzw. am Opsonin C3b. Es kann gesagt werden, dass Tef1p Plasminogen bindet, um einerseits die Degradierung von ECM-Proteinen zum Zweck der Gewebsinvasion zu erleichtern und andererseits die Degradierung von C3b als Mechanismus der Immunevasion durch verhinderte Erkennung durch phagozytierende Zellen zu verwenden. Ferner wird gezeigt, dass das Gen *TEF1* nicht essentiell für das Wachstum von *C. albicans* ist, da ein *tef1*-Deletionsstamm unter Standard-Kulturbedingungen die gleiche Wachstumsrate wie der Wildtyp zeigt. Ebenso zeigt sich keine Unterschiede im Wachstum im Vergleich zum Wildtyp, wenn der *tef1*-Deletionsstamm der Gegenwart von osmotischen und oxidativen Stressoren und Zellwand-störenden Substanzen ausgesetzt ist. Diese Effekte sind zurückzuführen auf die Anwesenheit des *TEF2*-Gens, welches in *C. albicans* ebenfalls für das Tef1-Protein kodiert. Dennoch sind *tef1*-Deletionsmutanten erhöht sensitiv gegenüber der antimykotischen Substanz Hygromycin, die direkt die Proteinsynthese in *C. albicans* inhibiert. *tef1*-Deletionsmutanten binden im Vergleich zum Wildtyp weniger C3d und Plasminogen. Daher resultiert die Deletion von *tef1* in einer geringeren Bindefähigkeit von *C. albicans* an Komplementproteine. Außerdem ist Tef1p an der Interaktion von *C. albicans* mit Neutrophilen beteiligt, da gezeigt wird, dass rekombinantes Tef1p an Neutrophile bindet und weiterhin *tef1*-Deletionsmutanten eine mangelnde Fähigkeit zur Auslösung der Produktion von Reaktiven Sauerstoffspezies (ROS) in Neutrophilen aufweisen.

Zusammenfassend kann festgehalten werden, dass mit dieser Arbeit die Bedeutung von Zelloberflächen-assoziierten Proteinen von *C. albicans* in der Virulenz, sei es für die Evasion des menschlichen Immunsystems oder die erleichterte Adhärenz an Zellen des Wirtes, gezeigt am Beispiel der Gpm1p und Tef1p, verdeutlicht wird. Die Aufklärung der Mechanismen, welche *C. albicans* in der Verwendung dieser Oberflächenproteine nutzt, kann

richtungsweisend für die Entwicklung neuer Strategien sein, mit denen dieser pathogene Pilz zukünftig noch besser bekämpft werden kann und dadurch die Lebensqualität der betroffenen Patienten verbessert werden kann.

1. INTRODUCTION

Humans are surrounded by and exposed to a variety of microorganisms everyday. Microorganisms can be beneficial or harmful. Beneficial microorganisms living in the human body aid in efficient production and acquisition of nutrients from food. Other beneficial microorganisms help in warding off harmful microorganisms which cause diseases. Disease-causing (pathogenic) microorganisms have the ability to infect humans that can eventually lead to death. It is with this reason that the human body is in a constant state of battle with microbial pathogens. Mechanisms to balance each other out have been developed by both humans and microbial pathogens through million years of co-evolution. On the one hand, microbial pathogens try to infect and overpower the human host using secreted and cell-surface components; but on the other hand, the human host uses mechanisms involving the immune system to counteract the different strategies microbial pathogens use. It is these complex interactions between pathogenic microorganisms and human host that researchers all over the world are trying to elucidate to gain further knowledge on how to effectively inhibit pathogenic microorganisms which could eventually lead to elimination of diseases and to improve the quality of life.

1.1. Human host

1.1.1. Immune system

The human immune system is composed of several physical, soluble and cell membrane-bound, and cellular components which make sure that the body is protected from foreign objects which can cause detrimental effects to the human body. The immune system's first line of defense are the physical barriers provided by the skin, inner surfaces of the body, their secretions and associated normal microflora, and mechanical mechanisms which protect the body from entry of unwanted microorganisms. In terms of humoral and cellular protection, the human body relies on the innate and adaptive arms of the immune system. The innate immune system is classically defined as non-specific response to foreign intruders of the human body. It mainly consists of the complement system, phagocytes (which include macrophages, neutrophils and dendritic cells), basophils and eosinophils, and natural killer (NK) cells. The complement system generates opsonins, anaphylatoxins and chemoattractants which induce the migration of phagocytes to the site of infection, inflammation, and formation of lytic pores. Adaptive immune system produces humoral and cellular specific responses to foreign objects present in the human body. Recently, it has been established that the innate immune system is closely related to the adaptive immune system through components of the complement system [1].

1.1.1.1 The Complement System

When the human body is intruded by foreign objects such as unwanted microorganisms, the first line of defense of the human immune system is the complement system. The complement system is a cascade of protein chain reactions which are composed of more than 40 soluble and cell surface-associated proteins. The main functions of the complement system are to mark foreign objects (opsonization) inside the human body for elimination by phagocytes, to elicit an inflammatory response at the site of infection, and to lyse specific cell types.

The complement system is activated by three different pathways. The first pathway is the spontaneous hydrolysis of C3 (alternative pathway, AP), the second pathway is induced by antibodies (classical pathway, CP), and the third is induced by mannose-binding lectins (MBL pathway). All of the three pathways converge to form the central complement molecule C3b. **Figure 1** shows the activation of the alternative pathway together with the components involved in the regulation and subsequent interaction with cells. CP and MBL pathway also produce the same cascade with a few differences.

C4 and C2 are the main starting components involved in the initiation of CP and MBL pathway. In the CP, C4 is cleaved by the C1qrs complex which is produced when C1q binds to Antigen-Antibody (Ag-Ab) complexes resulting in the autocatalysis of C1r, which then cleaves C1s. The cleaved C1s enzymatically cleaves C4 into C4a and C4b. C4b binds C2, which becomes susceptible to C1s and is cleaved into C2a and C2b. C4b2a complex is formed which is known as CP C3 convertase which cleaves C3 into C3a and C3b. C3b binds to the activating surface to form C4b2a3b complex. C4b2a3b complex functions as C5 convertase, which cleaves C5 into C5a and C5b.

In the MBL pathway, C4 and C2 activation can be achieved without antibody and C1 participation. The three proteins that initiate the MBL pathway are mannan-binding lectin/protein (MBL), and two mannan-binding lectin-associated serine proteases (MASP and MASP2). MBL binds to certain mannose residues on activating surfaces and subsequently interacts with MASP and MASP2. The MBL-MASP-MASP2 complex is similar to Ab-C1qrs complex of CP and leads to activation of C4, C2 and C3. The rest follows as in CP.

To avoid opsonization and lysis of normal human bystander cells near an area where complement is activated, the complement system employs several regulatory proteins which inhibit the deleterious action of the complement cascade. Complement regulatory proteins are either membrane-bound or soluble. The wide array of complement regulators act at the different stages of complement activation and activation pathways. Membrane-bound complement regulatory proteins include the complement receptors CR1 (CD35), CR2 (CD21) and CR3 (CD18/CD11b), complement receptor of the Ig superfamily (CRIg), decay accelerating factor (DAF, CD55), membrane

cofactor protein (MCP, CD46), and protectin (CD59). Soluble complement regulatory proteins include Factor H, Factor H-like protein 1 (FHL-1), Factor H-related protein 1 (CFHR1), C4b-binding protein (C4BP), properdin, carboxypeptidase N, C1q C1INH clusterin, vitronectin, and plasminogen. Specific mode of action of these proteins are comprehensively discussed in Zipfel *et al.* (2009) [3]. Factor H of the alternative pathway and C4BP of the classical pathway act as cofactors for Factor I. With the help of the cofactors, Factor I inactivates C3b by degrading it into fragment components, thereby inhibiting the amplification loop which then inhibits further production of C3b. CFHR1, on the other hand, acts at the C5 level. By binding to C5, CFHR1 inhibits the conversion of C5 into the anaphylatoxin and chemoattractant C5a and the terminal complement complex component C5b [6].

Vitronectin is also a regulator of the terminal complement complex. Vitronectin binds to specific components of the complex (i.e., C7 and C9) which thereby blocks the formation of the pore complex [2,4]. Plasminogen has recently been found to regulate the complement system by binding to C3 and degrading the opsonin C3b [9].

1.1.1.2. Neutrophils

Neutrophils are a part of the family of white blood cells which are responsible for making sure that foreign objects are eliminated. Neutrophils mostly blocks the detrimental effects of pathogenic microorganisms by phagocytosis. Neutrophils kill pathogens by producing antimicrobial proteins, engulfing foreign cells, exposing them to low pH and production of reactive oxygen species (ROS), and production of neutrophil extracellular traps (NETs) [7].

The activation of neutrophils has been comprehensively discussed in detail by Amulic *et al.* (2011) [8]. The main points are as follows. Neutrophils are activated by the presence of components of foreign objects such as microorganisms. Cytokines such as tumor necrosis factor (TNF)-alpha, and interleukins IL- β and IL-17 are produced when microbial components such as lipopolysaccharides (LPS) and formyl-methionyl-leucyl-phenylalanine (fMLP) are present. When cytokines and microbial components are present near the site of infection, endothelial cells express adhesion molecules such as P-selectin, E-selectin and intercellular adhesion molecules (ICAMs). When these adhesion molecules on the luminal side of endothelial cells are sensed

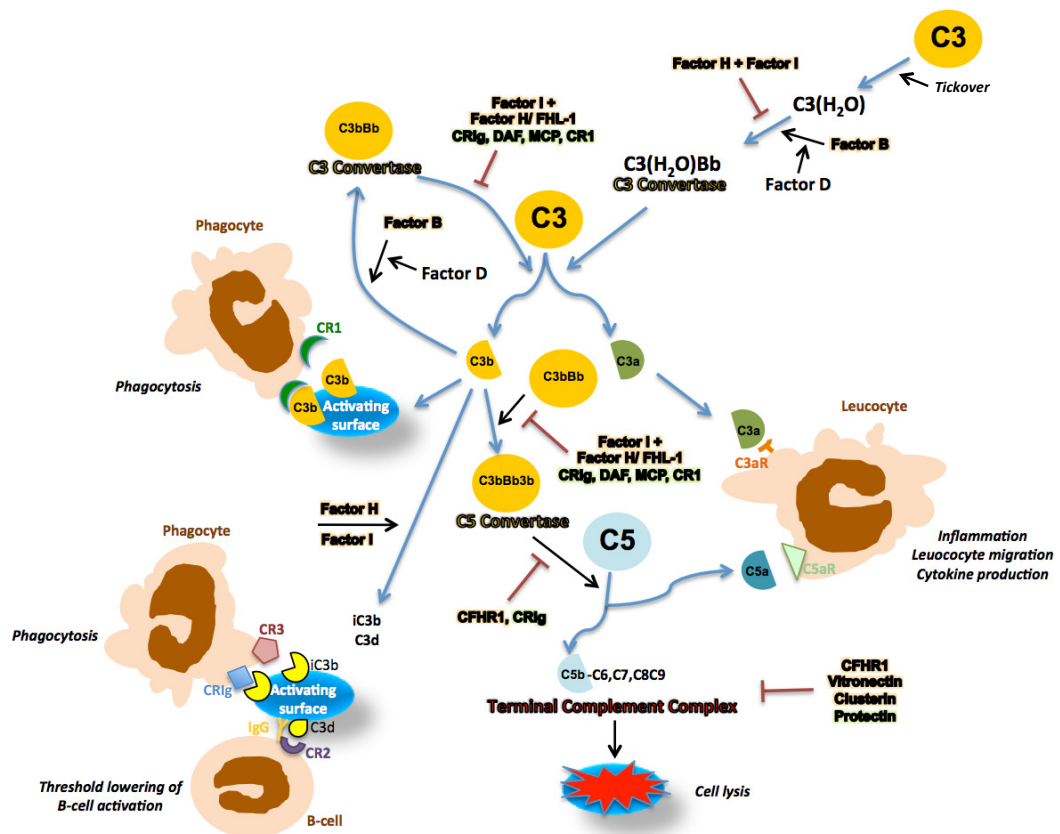


Figure 1. The alternative pathway activation of the complement system.

The alternative pathway is one of the three activation pathways of the complement system. It is activated by the spontaneous hydrolysis of the complement component C3 in the process called “tickover” producing C3(H₂O). Binding of Factor B to C3(H₂O) with subsequent cleavage to Bb by Factor D produces the C3 convertase. C3 convertase cleaves C3 into C3b and C3a. C3b participates in further production of more C3b by continuous binding of Bb to C3b producing the C3bBb convertase in the process called the “amplification loop”. C3b binds to activating surfaces and acts as ligand for different receptors on human phagocytes such as Complement Receptor 1 (CR1) and Complement Receptor of the Immunoglobulin superfamily (CRiG) for subsequent phagocytosis. Meanwhile, C3bBb is further used to form the C5 convertase (C3bBb3b) which cleaves C5 into C5b and C5a. C5a and C3a act as anaphylatoxins and ligands for C5a receptor (C5aR) and C3a receptor (C3aR), respectively, which produces inflammation, leucocyte migration and cytokine production. C5b binds C6, C7, C8 and C9 producing the terminal complement complex which forms pores on activating cell surfaces leading to lysis. The complement cascade is tightly controlled by several soluble and membrane-bound regulators. Soluble Factor H acts as co-factor for Factor I which degrades C3b to iC3b and further degradation of iC3b produces the C3d fragment. Factor H also binds to C3b to inhibit the convertase activity leading to its decay. Membrane-bound CR1 and CRiG also act as inhibitors of the C3 convertase together with Decay Accelerating Factor (DAF) and Membrane Cofactor Protein (MCP). In addition Complement Factor H-related Protein 1 (CFHR1) inhibits the C5 convertase and the formation of the terminal complement complex (TCC). Vitronectin, clusterin and protectin are also inhibitors of the TCC formation. Inactive C3b (iC3b), when bound to activating surfaces, can also be recognized by other phagocyte membrane-bound complement receptors such as Complement Receptor 3 (CR3) and CRiG also leading to phagocytosis. Moreover, when the IgG coreceptor Complement Receptor 2 (CR2) on the surface of B cells recognize C3d, the threshold of B cell activation is lowered.

by the neutrophils, they express receptors on their surface, such as P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin which allow them to “roll” and subsequently have a firm attachment on the endothelial cell surface by expressing $\beta 2$ integrins. The engagement of $\beta 2$ integrins with ICAMs initiates the change in neutrophils which include cell spreading and beginning of the oxidative burst process. The course of endothelial barrier traversal happens until the neutrophils reach the site of infection by following a chemoattractant gradient. Upon interaction of chemoattractants and other pathogen-associated molecular patterns (PAMPs) to their respective receptors on the surface of the neutrophils, a signalling cascade starts which mostly involve the MAPK/ERK pathway leading to engulfment, formation of phagolysosome, lowering of pH, production of reactive oxygen species, degranulation involving release of antimicrobials such as defensins, and/or formation of neutrophil extracellular traps (NETs).

Some of the well-studied neutrophil receptors are the complement and fMLP receptors, $\beta 2$ integrins and CD16 (Fc γ III receptor). Complement receptors (such as CR3) recognize iC3b-opsonized cells or debris. $\beta 2$ integrins recognize intercellular adhesion molecule 1 (ICAM-1). Other neutrophil receptors include the Toll-like receptors (TLRs). TLR4 recognizes LPS, while TLR2 recognizes bacterial lipopeptides. TLR5 and TLR9 bind flagellin and DNA, respectively.

Upon activation, neutrophils undergo respiratory burst where reactive oxygen species (ROS) are produced. ROS can modify and damage other molecules, making them effective microbial-killing substances. The ROS cascade begins when the NADPH oxidase complex assembles on the phagosomal and plasma membranes. When this happens, the molecular oxygen is reduced to superoxide. Superoxide can undergo several reactions producing different products. Rapid dismutation of superoxide leads to the formation of hydrogen peroxide while reaction of superoxide with nitric oxide forms the strong oxidant peroxynitrite. When myeloperoxidase (MPO) is released into the phagosome during degranulation, MPO can react with hydrogen peroxide to produce various reactive species, including hypochlorous acid. Hypochlorous acid is more reactive than superoxide which is thought to give it the capability to have direct antimicrobial effects in the phagosome.

1.1.2. Vitronectin

Vitronectin is a human soluble and membrane-bound glycoprotein of humans. It appears as 75- and 65-/10-kDa protein found in the plasma at a concentration of 200-400 μ g/ml. Vitronectin is produced mainly by the liver and is distributed to different organs through the blood. Vitronectin functions in the regulation of the complement system by inhibition of the terminal complement complex (TCC), cell adhesion, proliferation, cellular transport and an integral component of the extracellular matrix [10,11].

The structure of vitronectin is composed of the somatomedin-B (SMB) domain at the amino-terminus, followed by the Arg-Gly-Asp (RGD) motif, adjacent acidic epitope for high molecular weight kininogen binding, long connecting segment containing the collagen-binding domain, and the two hemopexin-like domains at the carboxy-terminus. The SMB domain contains the overlapping plasminogen activator inhibitor-1 (PAI-1) and the urokinase plasminogen activator/urokinase plasminogen activator receptor (uPA/uPAR) interaction sites. The RGD motif located directly adjacent to the SMB domain is responsible for the adherence to integrins on the human cell surface. The first hemopexin-like domain serves as specific binding site for infectious group A streptococci. The second hemopexin-like domain contains the basic heparin-binding site composed of two heparin-binding consensus sequences which serves as the primary, conformation-dependent, binding site of vitronectin for heparin and other glycosaminoglycans [12,13].

1.1.3. Plasminogen

Plasminogen is a 92-kDa single chain human plasma glycoprotein that circulates in plasma as an inactive proenzyme at a concentration of ca. 2 μ M. Plasminogen is primarily synthesized and secreted by liver cells. Plasmin as the key enzyme of the coagulation system dissolves preformed fibrin clots and regulates fibrinolysis and homeostasis [14,16]. Furthermore, plasmin also degrades components of the extracellular matrix and the basal membrane, including laminin, fibrin, and fibrinogen [15,17]. Another function of plasmin is the activation of matrix metalloproteases and elastase, which regulate wound healing, tissue remodeling, tumor metastasis, and angiogenesis [15,16].

Plasminogen is composed of an N-terminal preactivation peptide (ca. 8 kDa), five consecutive disulfide-bonded kringle domains (K1–K5), and a serine protease domain at the C-terminus. During activation, plasminogen is cleaved at specific sites to release the protease plasmin. Plasminogen is a precursor of the protease plasmin. In humans, plasminogen is activated and converted to plasmin by urokinase-type plasminogen activator and tissue-type plasminogen activator. It can also be activated by activators of bacterial origin such as staphylokinase from *Staphylococcus aureus* and streptokinase from *Streptococcus pneumoniae* [17]. Once activated, the serine protease degrades various substrates such as fibrinogen, proteins of the extracellular matrix [9,15], immunoglobulin G (IgG) [18], and complement component C3 [9,19].

1.2. Microbial pathogens

1.2.1. *Candida albicans*

Among pathogenic microorganisms, fungi are known to cause major human infections worldwide. An important pathogenic fungus which causes invasive fungal infections is *Candida albicans* [20,22]. *C. albicans* is a diploid fungus which belongs to the family Ascomycota. It is a commensal microorganism which lives in the gut, oral-pharyngeal, vulvo-vaginal areas, and on skin of humans. However, *C. albicans* persists, colonizes, and causes infection when the human host has an impaired immune system either because of having an acquired immunodeficiency syndrome, or receiving immunosuppressive drugs because of transplantation or chemotherapy. In addition, *C. albicans* also turns into a pathogen when human normal microflora becomes imbalanced and when the integrity of epithelial cells gets compromised [21].

As a pathogen, *C. albicans* causes both systemic and mucocutaneous infections in humans. *C. albicans* possesses several traits which enhance pathogenicity of the fungus. This includes the ability to evade the immune system, switch from yeast form to hyphae form, secrete proteases, and express adhesins and invasins. The morphotype switching is a well-known trait of *C. albicans* which gives it the ability to invade and exert pressure in destroying human cells when inside [22,25,26]. *C. albicans* is known to secrete proteases to degrade specific human host components to aid in the invasion and establishment of infection [23,24]. Adhesins and invasins, either secreted or cell wall-bound, mediate attachment and invasion of host cells [25,26]. Proteins are either expressed on the surface or secreted to give *C. albicans* the advantage of binding to complement regulatory proteins for immune evasion [27,29].

1.2.1.1. *Candida albicans* cell wall

C. albicans interacts with human host cells and soluble factors mainly through components of its cell surface. The cell wall architecture of *C. albicans* consists of two main layers. The outer layer is composed of mannans and glycoproteins while the inner layer contains chitin and β -1,3 glucan. The inner and outer layers are linked by the β -1,6 glucan. Mannans are found at the outer layer which are normally attached to proteins (mannoproteins). Also attached to the β -glucans are proteins which are either covalently or non-covalently bound. Glycophosphatidylinositol (GPI)-anchored proteins are attached to β -1,6 glucan while Pir (proteins with internal repeats) proteins are directly attached to β -1,3 glucan [28,30]. Non-covalently bound cell wall proteins are heterogeneously distributed on the cell surface. They may or may not be secreted, and they can be cell surface associated [29,31,32].

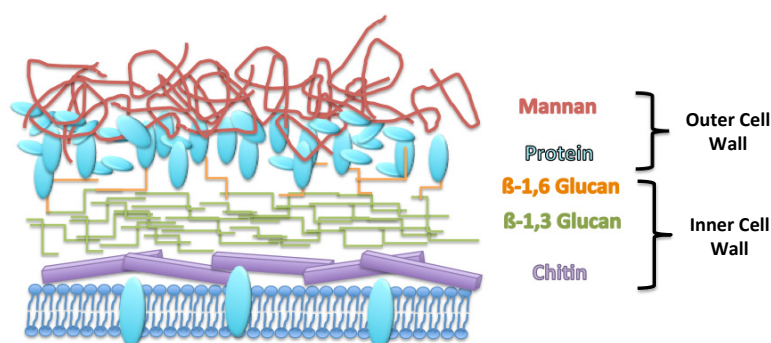


Figure 2. Cell wall components of *C. albicans*

The architecture of *C. albicans* cell wall is divided into two: inner and outer cell walls. The inner cell wall contains chitin and β -1,3 glucan. The outer cell wall is mainly composed of mannan and proteins. The outer and inner cell walls are connected by the β -1,6 glucan. The glucan components are also bound by cell wall proteins through covalent and non-covalent bonds.

Some proteins which are found on the *C. albicans* surface are non-conventional [30,33]. Non-conventional or “moonlighting” proteins play other roles aside from their main identified functions. Most of the non-conventional cell wall-associated proteins have major functions intracellularly. They do not have secretion signal peptides but are detected extracellularly either as secreted or surface-bound. Their functions range from attachment to human cells via the extracellular matrix proteins to binding of soluble human proteins [31,32].

Cell surface proteins which mediate *C. albicans* adhesion and invasion of host cells are called adhesins and invasins, respectively. Among the well-known adhesins and invasins, Als3 and Ssa1 are well-characterized. Als3 binds to the E- and N-cadherin on epithelial and endothelial cells, respectively [29,33]. Ssa1 is an additional cadherin ligand which *C. albicans* uses to invade human host cells [34]. Cell surface-associated proteins of *C. albicans* do not only bind host surface proteins but also soluble serum proteins. *C. albicans* binds serum proteins also for adhesion and invasion of human cells [29,37]. Among the moonlighting proteins, enolase is the well studied. It binds plasminogen and aids *C. albicans* to cross the blood-brain barrier in mice [38].

1.2.1.4. *Candida albicans* and the complement system

During systemic candidiasis, *C. albicans* is exposed to components of the human blood wherein soluble components of the immune system are present. Upon contact with the blood, *C. albicans* activates the human complement system [39,42]. *Candida albicans* activates the human complement system through the three complement activation pathways. Besides the normal complement system activation via the classical pathway through binding of anti-*Candida* antibodies and subsequent recognition by C1q, *C. albicans* mainly activates the complement system by the alternative pathway

[40,41,43]. Normally, intact blastoconidia of *C. albicans* is resistant to alternative pathway activation. However, β -glucans on the surface of *C. albicans* trigger the alternative pathway when mannan present on the outer layer of the cell wall becomes disrupted or blocked by anti-mannan antibodies, exposing the underlying glucans [42,44,45]. Specifically, β -1,6 glucan on the fungal cell surface triggers complement activation [43,46]. *C. albicans* also activates complement through mannose-binding lectin (MBL) pathway. The MBL pathway is important in opsonophagocytosis of yeast cells during hematogenous candidiasis as evidenced by the decreased C3 deposition on *C. albicans* and reduced phagocytosis by neutrophils in MBL-deficient sera [44].

Activation of the complement system leads to the generation of the opsonin C3b. Further action of the complement cascade produces C3b fragments iC3b and C3d [46]. *Candida albicans* binds these C3b fragments through components present on the fungal cell surface [47,50-52]. Most of the C3d and iC3b receptors on the surface of *C. albicans* were identified using antibodies against the human complement receptors CR2 [48,49,53] and CR3 [43,50-52], respectively. *Candida* Int1p binds iC3b [53-54] while several *C. albicans* surface proteins were isolated as C3d receptors based on their CR3-like characteristics. β -1,6 glucan also binds C3b/C3d [43].

Importance of the complement system during *C. albicans* infection was recently shown in that activated complement augments *C. albicans*-induced host cytokine production via C5a-C5aR signalling. Production of C5a augments production of pro-inflammatory cytokines IL-6 and IL-1 β but intracellular killing and phagocytosis are not affected [55]. In addition, the use of next-generation computational genetic analysis identified variations in *C1q*, *C1r*, *C1s* and their interaction with *C5* to be relevant in the survival of murine model during *C. albicans* infection, underscoring the role of the early complement classical pathway activation components in the resistance to fungal infection [56].

Aside from C5a, C3a also exerts antimicrobial effects against *C. albicans*. C3a binds to *C. albicans* cell surface and induces membrane perturbations and release of extracellular material. *C. albicans* isolates were found to induce complement degradation, leading to generation of C3a [57].

Complement regulatory proteins also bind *C. albicans* to enhance antimicrobial activity of neutrophils. Members of the Complement Factor H family proteins (CFH, CFH-like protein-1, CFH-related protein-1 and CFH-related protein-4) bind to *C. albicans*. CFH, CFHL-1 and CFHR1 increase attachment of neutrophils to *C. albicans*. Yeast-bound CFH and CFHR1 enhance generation of reactive oxygen species and release of antimicrobial protease lactoferrin by neutrophils [58].

1.2.1.5. Immune evasion of *C. albicans*

Pathogenic fungi such as *Candida albicans* and *Aspergillus fumigatus* are also known to bind regulatory proteins of the complement system to protect themselves from being recognized and opsonized by the effector cells of the innate immune system [35,36,59-61]. Regulatory proteins of the different pathways of the complement system are bound by fungal pathogens to inhibit further progress of the complement cascade. *C. albicans* takes advantage of the use of the complement regulatory proteins for its benefit to evade the complement system by binding several of these proteins. The complement inhibitors Factor H, FHL-1, CFHR1, and C4b-binding protein are bound by *C. albicans* which allow the fungus to inhibit the C3 conversion to the opsonin C3b, and stop the amplification loop which is responsible for the production of more C3b. Binding the terminal complement pathway inhibitor Factor H-related protein 1 by *C. albicans* can lead to the inhibition of the production of the anaphylatoxin and chemoattractant C5a, which may allow the fungus to avoid attack by phagocytes [62].

So far, four surface proteins of *C. albicans* have been identified to bind complement regulatory proteins of the complement system. Phosphoglycerate mutase (Gpm1p) [61], pH-regulated antigen 1 (Pra1) [62], high-affinity glucose transporter 1 (Hgt1p) [63] and recently, glycerol-3-phosphate dehydrogenase 2 (Gpd2) [65]. All of these proteins bind Factor H and the bound Factor H is active in acting as the cofactor of Factor I in C3b degradation which can lead to the inhibition of C3b deposition on the fungus, thereby inhibiting further recognition by phagocytes such as macrophages and neutrophils. Gpm1p and Pra1 bind to the short consensus repeats (SCRs) 6-7 and 19-20 of the Factor H molecule [61,62]. Among the four aforementioned proteins, Pra1 and Hgt1p also bind C4BP which inhibits the production of C3b from the classical pathway of complement activation [63,64]. Pra1 binds to the C4BP complement control protein domains 4, 7, and 8. In addition, Pra1 binds C3 to directly inhibit the activation of the complement cascade [66]. By directly binding to C3, Pra1 can inhibit the complement cascade leading to inhibition of production of the anaphylatoxins and chemoattractants C3a and C5a (Figure 3).

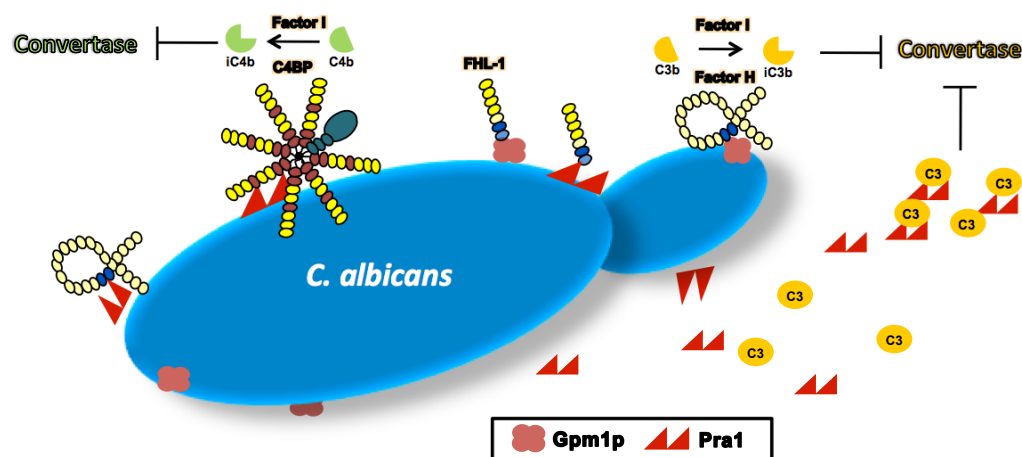


Figure 3. Complement evasion strategies of *C. albicans*.

C. albicans expresses Gpm1p and Pra1 to evade the human complement system. Surface Gpm1p and Pra1 bind the alternative pathway complement regulators Factor H and FHL-1 which act as cofactors for Factor I in inactivating the opsonin C3b producing iC3b. Pra1 binds the classical pathway regulator C4BP which also acts as cofactor for Factor I in converting C4b to iC4b. Inactivation of C3b and C4b leads to the inhibition of the C3 convertase. In addition, *C. albicans* secretes Pra1 which binds C3 to inhibit the production of C3b and the anaphylatoxins C3a and C5a.

Aside from binding complement regulatory proteins for immune evasion, *C. albicans* secretes proteases Saps 4-6 which interfere and inactivate host complement components C3b, C4b and C5. The secreted proteases also inhibit the terminal complement complex (TCC) formation. Secretion of complement degrading proteases provides a highly efficient complement defense response of this human pathogenic yeast that acts after the immediate acquisition of host complement regulators to the cell surface [23]. Secreted aspartic proteases also have a role in degrading the antimicrobial peptide Histatin-5. In doing so, *C. albicans* can persist in the oropharyngeal area and cause infection [24].

1.2.1.6. *Candida albicans* and vitronectin

Clinical isolates of *C. albicans* bind to vitronectin. *C. albicans* binding of vitronectin is optimized when the fungus is grown at 37°C and optimal binding is at pH 4 [67]. *C. albicans*-vitronectin binding is significantly inhibited by heparin, suggesting that the glycosaminoglycan-binding region of vitronectin is involved in the interaction [68]. Heat and protease treatments on *C. albicans* decrease the vitronectin binding capacity of the fungus indicating that the vitronectin receptor on the surface of *C. albicans* is a protein [67].

A 30-kDa molecule of *C. albicans* was identified to bind to vitronectin based on SDS and ligand blotting by radiolabelled vitronectin [68]. Other studies showed that both yeast cells and germ tubes of *C. albicans* have human integrin-like receptors which specifically bind vitronectin. *C. albicans* in the

yeast phase expresses vitronectin receptors which are antigenically related to the vertebrate $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins with molecular masses of 130, 110, 100, and 84 kDa [69-71]. *C. albicans* germ tubes have vitronectin receptors which are antigenically related to human α_v , β_3 and $\alpha_v\beta_3$, but not to $\alpha_v\beta_5$ [70]. The *C. albicans*-vitronectin interaction is believed to be an important aspect of both *C. albicans* virulence and human host protection of itself against fungal infection. On one hand, *C. albicans* binding to vitronectin increases the fungus' adherence to endothelial cells which could help in the dissemination of fungal cells during hematogenous infection or candidiasis and invasion of tissues during muco-cutaneous infection [69-71]. On the other hand, vitronectin increases the attachment of *C. albicans* to macrophages which could help in a more effective phagocytosis of the fungal cells [68].

1.2.1.7. *C. albicans* and plasminogen

C. albicans binds plasminogen and bound plasminogen is activated to plasmin by human uPA or tPA as the fungus does not produce any plasminogen activators. Plasmin, when bound to *C. albicans* degrades fibrinogen [37]. At present, there are 11 *C. albicans* surface proteins which bind plasminogen as listed in **Table 1**. The binding of the enumerated *C. albicans* surface proteins to plasminogen involve lysine residues.

Table 1. Plasminogen-binding surface proteins of *C. albicans*

Name	Reference
Phosphoglycerate mutase (Gpm1p)	[37,61]
pH-regulated antigen 1 (Pra1)	[37,62]
Translation elongation factor 1 (Tef1p)	[37]
Catalase	[37]
Thioredoxin peroxidase	[37]
Alcohol dehydrogenase (Adh1)	[37]
Glyceraldehyde-3-phosphate dehydrogenase (Gapdh)	[37]
Phosphoglycerate kinase (Pgk1)	[37]
Fructose biphosphate aldolase (Fba1)	[37]
Enolase (Eno1)	[38]
glycerol-2-phosphate dehydrogenase (Gpd2)	[65]

1.2.1.2. *Candida albicans* phosphoglycerate mutase (Gpm1p)

Phosphoglycerate mutase (Gpm1p) is a 27-kDa, cytoplasmic and cell surface-associated protein of both yeast and hyphal forms of *C. albicans* [61,72]. As a cytoplasmic protein, Gpm1p converts 3-phosphoglycerate to 2-phosphoglycerate during glycolysis and vice versa during gluconeogenesis. On the cell surface, Gpm1p binds the host complement Factor H, FHL-1 and plasminogen [61]. Binding of Factor H and FHL-1 by Gpm1p is a complement

evasion strategy of *C. albicans*. Presence of anti-Gpm1p antibodies in serum of human candidiasis patients indicate that Gpm1p is an immunogenic protein [73].

Gpm1p is encoded by the gene *GPM1* (orf19.903). Expression of *GPM1* is regulated by several conditions. *GPM1* is downregulated when *C. albicans* is exposed to farnesol [74]. In addition, different antifungal agents have varying effects on the expression of *GPM1*. *GPM1* mRNA increases during fluconazole exposure, but is downregulated when exposed to ketoconazole [75]. *GPM1* gene is also regulated by *C. albicans* growth phase. *GPM1* mRNA levels increase during exponential growth phase of the yeast form, then decrease to relatively low levels in stationary phase. *GPM1* mRNA levels then decrease rapidly after dilution of late-exponential cells into fresh hyphal-inducing media [76]. Moreover, the *GPM1* transcript is induced in biofilm-versus-planktonic cell culture [61,77]. *GPM1* expression is also upregulated in a dual-species biofilm with *S. aureus* [78]. Morphogenetic transcriptional regulators also affect the expression of *GPM1*. *GPM1* is regulated by the transcription factor Efg1p but not its homologue Efh1p. Furthermore, upregulation of *GPM1* occurs during amino acid starvation which is dependent on *Gcn4p*, a transcriptional activator of amino acid biosynthetic gene. Moreover, deletion of *GPM1* gene renders *C. albicans* unable to grow in gluucose. *gpm1* deletion mutant grows only on ethanol and/or glycerol as carbon source/s [61].

1.2.1.3. *Candida albicans* translation elongation factor 1-alpha (Tef1p)

Translation elongation factor 1-alpha (Tef1p) is a canonically important protein in eukaryotic protein synthesis. Tef1p binds guanosine-5'-triphosphate (GTP) and binds to aminoacyl transfer RNAs (tRNAs). The Tef1p-GTP-tRNA complex binds to the ribosome delivering the amino acids contributing to the elongation of the polypeptide.

C. albicans Tef1p is a 50-kDa protein which is found in both cytoplasmic and cell surface components of the fungus [79]. Aside from its role in protein synthesis in the cytoplasm, cell-surface Tef1p binds to plasminogen [37]. Tef1p protein is encoded by two *C. albicans* genes *TEF1* and *TEF2* [80]. The *TEF1* gene is upregulated in reconstituted human oral epithelium (RHE) model of oral candidiasis and in clinical oral candidiasis isolates [81,83,84]. In addition, *TEF1* is induced upon phagocytosis by macrophage-like cells [82]. *TEF1* is thought to be essential as no *TEF1* disruptants were obtained by UAU1 method using the BWP17 strain [87].

1.2.2. Complement evasion by bacterial pathogens

Microbial pathogens have evolved immune evasion strategies to further the infection process. Immune evasion strategies by pathogens vary from masking themselves by binding host proteins to degrading host components by secreting degradative proteins. Bacteria such as *Staphylococcus aureus*

and *Neisseria meningitidis* are well known to evade the immune system which is the reason for their prevalence in nosocomial infections and leading cause of deaths in humans. By possessing secreted and cell wall-bound proteins, *S. aureus* can block the three activation pathways of the complement system. Sbi [83,84], SCIN [85,86], Efb [84] and SSL-7 [89,90] are just some of the staphylococcal proteins which are effective inhibitors of the specific parts of the complement cascade either by binding complement components or degrading them. *N. meningitidis* binds Factor H to act as a cofactor for Factor I in inactivating the opsonin C3b [91,92].

1.2.3. Bacterial pathogens and vitronectin

Microbial pathogens utilize the various functions of vitronectin for their proliferation inside the human body. The terminal complement complex (TCC) inhibition and adhesive properties of vitronectin make it a useful human host protein for microbial pathogens in evading the immune system and invading host tissues. Bacterial pathogens such as *Haemophilus influenzae* [93-95], *Haemophilus ducreyi* [96], *Neisseria meningitidis* [97], and *Moraxella catarrhalis* [98-99] have serum resistance due to their ability to bind vitronectin from human plasma. Bound vitronectin on the surface of the bacterial cells inhibits the formation of the TCC which prevents the lysis of the cells [2,3].

In addition, bacterial pathogens bind vitronectin to attach to human cells for subsequent colonization and invasion. *Escherichia coli*, *S. aureus* and *Streptococcus pneumoniae* utilize the adhesive property of vitronectin to attach to different types of host cells [100]. Adhesins on the surface of these bacterial pathogens bind to the heparin-binding domains on the vitronectin molecule which, in turn, binds to host cells through the integrin-binding Arg-Gly-Asp (RGD) motif. As an example, binding of vitronectin to gain access to epithelial and endothelial cells is a mechanism employed by *S. pneumoniae*. When vitronectin is bound to the integrin $\alpha_v\beta_3$ on host cells via the RGD motif, *S. pneumoniae* can bind to vitronectin through its heparin binding domains. By binding to the heparin binding domain of vitronectin, *S. pneumoniae* can trigger its internalization by activating the signalling pathway which involves the integrin-linked kinase (ILK), phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) [101].

1.2.4. Bacterial pathogens and plasminogen

Plasminogen is utilized by microbial pathogens for tissue invasion by degrading components of extracellular matrix [102], and for immune evasion by degrading the opsonin C3b. Bacterial pathogens like *Leptospira interrogans* [103-105], *Borrelia burgdorferi* [106], *Salmonella enterica* serovar Typhimurium [107], and *Streptococcus pneumoniae* [108] bind plasminogen to degrade components of the extracellular matrix. The bound plasminogen is activated either by the human plasminogen activators or produced by the bacteria themselves [17]. The bound plasmin maintains its activity in

degrading substrates. Recently, it has been shown that pathogens bind plasminogen, not only for tissue invasion, but also for evasion of the complement system. Bacteria-bound plasminogen, when converted to plasmin, binds to C3 and C3b and degrades them, thereby avoiding recognition by phagocytes. This mechanism of immune evasion has been shown for bacterial pathogens *S. aureus* [84,109], *Bacillus anthracis* [11] and *Francisella tularensis* [18] but not for fungal pathogens.

1.3. Objectives of the study

In this dissertation two of the virulence mechanisms of *C. albicans* were investigated: evasion of the innate immune system, and attachment to human host cells. It was therefore the aim of this thesis to elucidate the roles of specific *C. albicans* surface proteins in the virulence and immune evasion of the fungus. In this study, two fungal proteins were selected and characterized in detail.

Specifically, this thesis aimed to:

- Determine human host ligand for *C. albicans* Gpm1p
- Elucidate the function and mechanism of *C. albicans* Gpm1p-human host ligand interaction
- Clone, express and purify recombinant *C. albicans* Tef1p
- Determine human host ligand for *C. albicans* Tef1p
- Elucidate the function and mechanism of *C. albicans* Tef1p-human host interaction

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Human sera and cells

Normal human serum (NHS) samples were obtained from healthy laboratory personnel. Sera were stored at -80°C prior to use. Complement was inactivated from the serum either by adding ethylenediaminetetraacetic acid (EDTA) to a final concentration of 10 mM (NHS-EDTA) or by heat inactivation at 56°C for 30 min (hiNHS). Human umbilical vein endothelial cells (HUVEC) and keratinocytes (HaCaT) cells were purchased from ATCC (Vanassas, VA, USA). The use of human neutrophils in this study was conducted according to the principles expressed in the Declaration of Helsinki. All protocols used in this study were approved by the local ethics committee of the University of Jena under the permit no. 2207-01/08. Written informed consent was provided by all study participants. Neutrophils were isolated from whole blood using PolymorphPrep (Axis-Shield PoC AS, Oslo, Norway) gradient centrifugation using the manufacturer's instructions.

2.1.2. Chemicals, reagents and plastic materials

Unless specified otherwise, chemicals and reagents were purchased in the highest available quality from Sigma or Roth. RPMI1640 medium, Dulbecco's modified eagle medium (DMEM), and Dulbecco's phosphate buffered saline (DPBS) were from Lonza (Verviers, Belgium). MaxiSorp and PolySorp microtiter plates were manufactured by Nunc, New York, NY, USA.

2.1.3. Microbial media and supplements

E. coli cells were cultivated in sterile LB (Luria Broth) medium (10 g bacto-tryptone, 5 g bacto-yeast extract, 10 g NaCl, pH adjusted to 7.2) or LB agar plates (LB medium + 15 g/l agar). To select for colonies containing the desired plasmid, LB medium was supplemented with 50-100 $\mu\text{g/ml}$ ampicillin (Invitrogen).

Media used for *C. albicans* cultivation were YPD broth [2% glucose, 2% peptone, 1% yeast extract; in w/v]. *C. albicans* GPM1 strains were YPGE broth [3% glycerol, 2% ethanol, 2% peptone, 1% yeast extract; in w/v] or SG broth [3% glycerol, 2% peptone from casein, 1% yeast extract; in w/v]. Agar media were prepared as above with addition of 1.5% (w/v) agar.

For selection purposes *C. albicans* cells were spread on SD agar (2% dextrose, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% agar) supplemented with 20 mg/ml arginine, histidine and/or uridine as required.

2.1.4. Equipment

The laser scanning microscopes used were LSM 510 META and LSM 710 (Zeiss, Jena, Germany). Flow cytometry was performed by BD LSR II (Becton Dickinson, Heidelberg, Germany). The ELISA reader used was Multiskan Ascent (Thermo Scientific, Thermo Labsystems, Finland). The Biacore 3000 instrument and sensor chips were from Biacore AB, Uppsala, Sweden. Äkta purifier and Äkta FPLC used for protein purifications were from GE Healthcare Life Sciences.

2.1.5. Purified and recombinant proteins

Plasma proteins Factor H, Factor I, C3, C3a, C3b, C3c, C3d and iC3b were purchased from Complement Technology, Inc. (Texas, USA). C4BP was provided by Prof. Dr. Ana Blom (Department of Laboratory Medicine, Malmö University Hospital, Sweden). Recombinant proteins Efb, C3d and C3dg were provided by Sascha Böhm (Department of Infection Biology, HKI, Jena, Germany). Plasminogen and tissue-type activator (tPa) were purchased from Haemochrom Diagnostica GmbH (Essen, Germany). Extracellular matrix proteins fibronectin, laminin, fibrinogen, collagen I, collagen III and collagen IV were purchased from Calbiochem while vitronectin was from Becton Dickinson.

2.1.6. Antibodies

Primary antibodies used in immunodetection methods were either generated in this study or bought as specified in Table 2. HRP-coupled secondary antibodies (**Table 2**) used for enzyme-linked immunosorbent assay (ELISA) and Western blotting experiments were purchased from Dako Denmark (Glostrup, Denmark). Fluorescent dye-labelled secondary antibodies (**Table 2**) used in flow cytometry and laser scanning microscopy experiments were purchased from Invitrogen Molecular Probes (Life Technologies GmbH, Darmstadt, Germany).

Table 2. Antibodies used in this study.

Primary antibodies		Secondary antibodies
Name	Specificity	Name
Rabbit Gpm1p antiserum (this study)	Gpm1p (polyclonal)	Alexa 647 [®] -conjugated rabbit anti-goat
Rabbit vitronectin antiserum (Complement Technology)	Vitronectin (polyclonal)	Alexa 647 [®] -conjugated goat anti-mouse
Mouse fibronectin antibody (Sigma-Aldrich)	Fibronectin (monoclonal)	Alexa 488 [®] -conjugated goat anti-rabbit
Mouse laminin antibody (Sigma-Aldrich)	Laminin (monoclonal)	Alexa 647 [®] -conjugated rabbit anti-goat
Rabbit fibrinogen antiserum (Calbiochem)	Fibrinogen (polyclonal)	Alexa 488 [®] -conjugated goat anti-rabbit
Mouse collagen I antibody (Sigma-Aldrich)	Collagen I (monoclonal)	HRP-coupled goat anti-rabbit
Mouse collagen III antibody (Sigma-Aldrich)	Collagen III (monoclonal)	HRP-coupled rabbit anti-goat
Mouse collagen IV antibody (Sigma-Aldrich)	Collagen IV (monoclonal)	HRP-coupled goat anti-mouse
Mouse Gpm1p antibody (this study)	Gpm1p (monoclonal)	
Mouse vitronectin antibody (Acris Antibodies GmbH)	Vitronectin (monoclonal)	
Mouse penta-His antibody (Qiagen)	His-tag (monoclonal)	
Goat Factor H antiserum (Complement Technology)	Factor H (polyclonal)	
Rabbit C4BP antiserum (Prof. Ana Blom)	C4BP (polyclonal)	
Rabbit C3d antiserum (Dako)	C3d (polyclonal)	
Goat C3 antiserum (Complement Technology)	C3 and C3b (polyclonal)	
Goat plasminogen antiserum (Acris Antibodies GmbH)	Plasminogen (polyclonal)	
Rabbit Tef1p antiserum (this study)	Tef1p (polyclonal)	

2.2. Methods

2.2.1. Microbiological methods

2.2.1.1 Strains used

Pichia pastoris

Pichia pastoris X33 was used to produce the recombinant His-tagged *C. albicans* Gpm1p described in Poltermann *et. al* (2007) [61].

Escherichia coli

One Shot® TOP10 competent *E. coli* (Invitrogen) that lacks T7 RNA polymerase was used for characterization, propagation and maintenance of the plasmid construct for cloning the *C. albicans* *TEF1* gene. The *E. coli* expression strain BL21 Star™ (DE3) (Invitrogen) was used for protein expression. *E. coli* DH5α was used to propagate plasmids used in cloning, deletion and reintegration of *TEF1* in *C. albicans*.

Candida albicans

The *C. albicans* strains used and generated in this study and their corresponding genotypes and descriptions are listed in **Table 3**.

2.2.1.2. Cultivation and conservation of microbial strains

During the cloning experiments, *E. coli* strains were grown in LB medium at 37°C overnight with shaking at 180 rpm. For use in various experiments, *C. albicans* *TEF1* strains were grown in YPD broth overnight in a rotary shaker at 180 rpm at 30 °C. *C. albicans* *GPM1* strains were grown in SG or YPGE broth medium at 180 rpm at 30 °C. Stock cultures of generated *C. albicans* *TEF1* strains were stored at -80 °C in YPD broth with 20% glycerol. Cultures for experiments were maintained on YPD or SG agar plates at 4 °C and colonies were restreaked on fresh agar plates every 2 weeks.

2.2.1.3. *C. albicans* growth curves in liquid and agar media

Aliquots of YPD broth overnight cultures of *C. albicans* *TEF1* strains were washed twice in phosphate buffered saline (PBS) and cells with optical density at 600 nm ($OD_{600\text{ nm}}$)=0.1 were inoculated in YPD broth (for liquid culture growth curve) or 10-fold serial dilutions in 5 µl (covering a range of 10^6 – 10^1 cells) were spotted onto YPD agar (for solid medium growth). Liquid culture growth curve experiment flasks were incubated for 14 h at 30 °C with collection of samples every hour for the measurement of optical density at 600 nm ($OD_{600\text{ nm}}$). YPD agar plates were incubated for 2-3 days at 30 °C or 37 °C.

2.2.1.4. *C. albicans* stress susceptibility

Aliquots of YPD broth overnight cultures of *C. albicans* *TEF1* strains were washed twice in phosphate buffered saline (PBS) and 10-fold serial dilutions in 5 ml (covering a range of 10^6 – 10^1 cells) were spotted onto YPD agar containing calcofluor white (6.7 nM), congo red (20 µg/ml), SDS (0.004%), NaCl (1.5 M), sorbitol (1.0 M), menadione (25 µM), H₂O₂ (2 µM), t-BOOH (2 mM); and hygromycin (0.5 mg/ml). Plates were incubated for 2-3 days at 30 °C.

Table 3. *C. albicans* strains used in this study.

Strain	Genotype	Reference
SC5314	Wild type	[111]
<i>gpm1</i> Δ	<i>gpm1::hisG-URA3-hisG/GPM1</i> , <i>ura3::imm434/ura3::imm434</i>	[61]
<i>gpm1</i> Δ/Δ	<i>gpm1::hisG/gpm1::hisG-URA3-hisG</i> , <i>ura3::imm434/ura3::imm434</i>	[61]
<i>gpm1</i> Δ/Δ::GPM1	<i>gpm1::hisG/GPM1::hisG-URA3-hisG</i> , <i>ura3::imm434/ura3::imm434</i>	[61]
BWP17	<i>ura3::λimm434/ura3::λimm434</i> <i>arg4::hisG/arg4::hisG his1::hisG/his1::hisG</i>	[112]
<i>tef1</i> Δ	<i>TEF1/tef1::ARG4</i>	This study
<i>tef1</i> Δ/Δ <i>ura</i> ⁺	<i>tef1::ARG/tef1::HIS</i>	This study
<i>tef1</i> Δ/Δ	<i>tef1::ARG/tef1::HIS+Clp10 (URA)</i>	This study
<i>tef1</i> Δ/Δ::TEF1	<i>tef1::ARG/tef1::HIS+Clp10+TEF1 (orf19.1435, URA)</i>	This study
BWP17+Clp30	<i>ura3::λimm434/ura3::λimm434</i> <i>arg4::hisG/arg4::hisG his1::hisG/his1::hisG + Clp30</i>	[113]

2.2.2. Molecular Biological Methods

2.2.2.1. *C. albicans* TEF1 cloning

The *TEF1* gene was amplified from the chromosomal DNA of *C. albicans* SC5314 isolated using MasterPure™ yeast DNA purification kit (Epicentre Biotechnologies). Production of blunt-end polymerase chain reaction (PCR) product was done through amplification of *TEF1* from the genomic DNA template using the primers: CaTef1pET101For and CaTef1pET101Rev (Table 4) and the program: initial denaturation, 98 °C for 30 sec; denaturation, 98 °C for 10 sec; annealing, 65 °C for 30 sec, elongation, 72 °C for 45 sec; and final extension, 72 °C 10 min; cycles, 30. The following recipe was used for 20-µl reaction: DNA template, 0.4 µl; 10mM DNTPs, 0.4 µl; 2.5 µM forward primer, 0.5 µl; 2.5 µM reverse primer, 0.5 µl; 5X HF Buffer, 4 µl; Phusion polymerase, 0.2 µl; and water, 14 µl. Then TOPO cloning reaction was done to insert blunt-end PCR product to the expression plasmid pET101/D-TOPO® using manufacturer's instructions. Briefly, 2 µl of successfully amplified *TEF1* PCR products were mixed with 1 µl NaCl, 2 µl sterile water and 1 µl TOPO

vector, and incubated for 5 min, before the reaction mixture was transformed into One Shot TOP10 *E. coli* competent cells. Transformation of pET101-CaTef1 in *E. coli* competent cells was done using manufacturer's instructions. Colony PCR of grown colonies to select positive transformant was done using Quiagen HotStar Taq Master Mix Kit using the following recipe: 12.5 µl HotStar Taq Master Mix, 0.625 µl of 10 µM forward primer, 0.625 µl of 10 µM reverse primer, and 10.25 µl water. Program for colony PCR used was as follows: initial denaturation, 95 °C for 15 min; denaturation, 94°C for 1 min; annealing, 65°C for 1 min; elongation, 72 °C for 1 min; and final elongation, 72 °C for 10 min; cycles, 30. PCR products were analyzed by agarose gel electrophoresis and positive colony was purified and conserved. Transformed *E. coli* cells with correct pET101-CaTef1 were then cultivated on LB medium containing 50 µg/ml ampicillin for 24 h at 37 °C. *TEF1*-containing clones were cultivated for plasmid isolation.

Plasmid isolation was done by cultivating *E. coli* transformants harboring pET101-CaTef1 in 3 ml LB media (supplemented with ampicillin) overnight at 37°C with shaking. Plasmids were isolated using Spin Plasmid Mini Two Kit (Invitex) following the manufacturer's instructions. Briefly, cells were harvested, resuspended in 250-µl resuspension buffer, and lysed by lysing buffer. Thereafter a neutralization buffer was added, and the mixture was centrifuged at 3000 x *g* for 10 min. The supernatant was applied to a QIAprep spin column, and the plasmid DNA was bound to the column through centrifugation at 3000 *g* for 30 s. Before elution of the plasmid DNA in 30 µl water, the column was washed with wash buffer.

Transformation into *E. coli* BL21 Star (DE3) expression strain was done by following manufacturer's instructions. Briefly, 10 ng plasmid DNA (in 2 µl water) were added to thawed BL21 Star cells, and incubated for 30 min on ice. After heat-shock, the cells were incubated in SOC medium (Invitrogen), and plated on LB plates containing ampicillin. Positive transformants were screened using colony PCR as described above.

2.2.2.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to analyze amplified DNA fragments. Samples were mixed with 6 x DNA loading dye solution (Fermentas), and separated using 1% TBE (890 mM tris, 890 mM boric acid, 20 mM EDTA) agarose gel containing 0.1 µg/ml ethidium bromide. SmartLadder (Eurogentec) was used as marker. DNA bands were visualized under UV light in a gel documentation system (BioRad).

2.2.2.3 DNA sequencing

In order to confirm that *TEF1* was in frame with the C-terminal His-tag, and to exclude mutations in the sequence, plasmids containing the construct were sequenced via Sanger sequencing using an ABI PRISM® 3130x Genetic Analyzer (Applied Biosystem).

2.2.2.4. Southern blotting

Ten µg of chromosomal DNA of *C. albicans* SC5314 and *TEF1* deletion strains were cut with *PciI*. DNA fragments were separated on a 1% (wt/vol) agarose gel and blotted onto a Hybond N⁺ nylon membrane (GE Healthcare, Freiburg, Germany). Labeling of the DNA probe was performed using the PCR digoxigenin (DIG) labeling mix (Roche Applied Science, Mannheim, Germany). For hybridization and detection of DNA-DNA hybrids, the DIG Easy Hyb and the CDP-Star ready-to-use kit (Roche Applied Science, Mannheim, Germany) were used according to the manufacturer's instructions.

2.2.2.5. *C. albicans TEF1* deletion

The generation of *TEF1* knock-out and reintegration strains were done under the supervision of Pedro Miramón (Department of Molecular Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research and Infection Biology, HKI). The *tef1Δ/Δ* homozygous null mutant was generated using a PCR-based gene disruption technique [116]. Starting with the Arg-, His- and Ura-auxotrophic parental strain BWP17, the complete open reading frames (ORFs) of both *TEF1* alleles were replaced with polymerase chain reaction (PCR)-amplified *ARG4* and *HIS1* disruption cassettes flanked by 104 base pairs of target homology region. Two sequential transformations using the improved lithium-acetate method were applied for both disruption cassettes. Primers *TEF1*-KO _{fwd} and *TEF1*-KO _{rev} (**Table 4**) were used for generation of the *ARG4* and *HIS1* deletion cassettes with the pFA-*ARG4* and pFA-*HIS1* plasmids as templates. Resultant deletion cassettes were used to sequentially delete both copies of *TEF1* (orf19.1435). The resultant Ura-auxotrophic mutant was rendered prototrophic for uridine by transformation with the NcoI-linearized plasmid Clp10, which harbors the *URA3* gene and stably integrates at the *RPS10* locus. The correct deletion of both alleles and integration of Clp10 was verified by colony PCR using target gene and disruption/integration cassette flanking and internal primers: *TEF1*-disrpt _{nup}, *TEF1*-disrpt _{ndown}, *ARG4*-F1, *ARG4*-R1, *HIS1*-F1, *HIS1*-R1, *URA3*-F2 and RPF-F1 (**Table 4**), respectively.

Additionally, Southern blot analysis using a 517-base-pair PCR product, generated with the primers CaTEF1Int-Rev and CaTEF1Int-Fwd from *C. albicans* SC5314 genomic DNA, as a probe on *PciI*-digested genomic DNA was used to confirm deletion of *TEF1*.

For the generation of a *tef1Δ/Δ::TEF1*-reconstituted strain, the open reading frame of *TEF1* as well as 406 base pairs of upstream and 2,145 base pairs of downstream sequence were amplified from SC5314 genomic DNA with the Phusion High-Fidelity DNA Polymerase Kit (Finnzymes) using the *Sall* restriction site-containing primer CaTEF1Complmnt-Fwd and *MluI* restriction site-containing primer CaTEF1Complmnt-Rev (**Table 4**). The resulting PCR product was first subcloned into pCR2.1 for easy digestion generating pCR-*TEF1*. The plasmid pCR-*TEF1* was screened for right insertion by digesting

with NcoI and Sall restriction enzymes. pCR-TEF1 was then digested with Sall, MluI and PstI. PstI was additionally used to distinguish the *TEF1* insert and the rest of the plasmid since the Sall-MluI digested plasmid has two fragments of the same size. The digested pCR-TEF1 was then further purified with the QIAquick PCR Purification Kit (Qiagen). In parallel 0.3 µg/µl of plasmid Clp10 was digested with Sall and MluI restriction enzymes then heat inactivated by an incubation at 65 °C for 20 min. The linearized plasmid was dephosphorylated with calf intestinal alkaline phosphatase (New England BioLabs) and gel extracted using the QIAquick Gel Extraction Kit (Qiagen). The *TEF1* insert and Clp10 vector were then ligated for 30 min at 22 °C using the Rapid DNA Ligation Kit (Fermentas). Five µl of ligation product was used for the transformation of *E. coli* DH5α and positive clones were selected on LB agar plates supplemented with 50 mg ml⁻¹ Ampicillin. Plasmid Clp10 carrying *TEF1* was re-isolated using plasmid miniprep (peqlab) and midiprep (Qiagen) kits and confirmed by control digestions with Sall and MluI and sequencing. The final plasmid was then digested with StuI prior to transformation into the uridine auxotrophic *C. albicans* strain *tef1Δ/Δura⁻*. Positive clones were selected on SD agar plates without amino acids. Correct integration was verified by PCR on whole yeast colonies using primers RPF-F1 and URA3-F2.

Table 4. Primers used in this study.

Primer	Sequence (5'→3')	Reference
CaTef1pET101For	caccatgggtaaagaaaaaactcac	This study
CaTef1pET101Rev	tttctagcagctttttgagcagc	This study
TEF1KO _{fwd}	aaagtaaagtaaagaaaaaaatttccctcgatcttgatttta cttctcttcttcttcttcttcttcttcacacattatagtcataatcaatc <u>gaagcttcgtacgctgcaggtc</u>	This study
TEF1KO _{rev}	ttttttaaataatataatttcaaatataataagaaaataaaactaa ataaatgaatacaaaaataaaaacttaacagatcttacataatattc <u>aactagctctgatatcatcgatgaattcgag</u>	This study
TEF1-disrptup	ttttgggtttttgtgttt	This study
TEF1-disrptdown	ttcaaaacaaccggactac	This study
TEF_2	aatggtttgtcgggtggtctggt	This study
TEF_3	gaaccatccaccaactgtccat	
CaTEF1Int-Fwd	gacgctattgaaccacca	This study
CaTEF1Int-Rev	ccatcttgacgatagca	This study
CaTEF1Complmnt-Fwd	aaaaaagtcgacgctgatacggcatgccag	This study
CaTEF1Complmnt-Rev	aaaaaaacgcgttattagcaacttagacaatccctg	This study
ARG4-F1	ggatatgttggtactgatttagc	[113]
ARG4-R1	aatggatcagtggcaccggtg	[113]
HIS1-F1	ggacgaattgaagaaagctggtgcaaccg	[113]
HIS1-R1	caacgaaatggcctcccctaccacag	[113]
URA3-F2	ggagttggattagatgataaaggatgatgg	[113]
RPF-F1	gagcagtgtacacacacacatcttg	[113]

Underlined nucleotides of TEF1KO_{fwd} and TEF1KO_{rev} primers indicate pFA-ARG4 and pFA-HIS1 annealing regions; underlined nucleotides of primer CaTEF1Complmnt-Fwd indicate Sall restriction site and of primer CaTEF1Complmnt-Rev indicate MluI restriction site.

2.2.3. Protein Biochemistry and Immunological Methods

2.2.3.1. ELISA binding studies

Binding of extracellular matrix (ECM) proteins to Gpm1p

To determine the binding of different extracellular matrix (ECM) proteins to Gpm1p, the enzyme-linked immunosorbent assay was used. A 96-well microtiter plate was coated with 0.25 µg of Gpm1p or human serum albumin (HSA) in carbonate-bicarbonate buffer overnight at 4 °C. After washing one time with distilled deionized water and one time with the washing buffer (phosphate-buffered saline + 0.05% Tween 20), the plate was blocked with the blocking solution (4% milk powder + 2% bovine serum albumin in phosphate-buffered saline) at room temperature (RT) for 2 hour. The plate was then washed two times with washing buffer. Seven hundred fifty ng of ECM proteins (fibronectin, vitronectin, laminin, fibrinogen, collagen I, collagen III or collagen IV) were added to the plate and incubated for 1 hour at RT. After washing two times with washing buffer, the corresponding primary antibody (1:1000 in blocking solution) as listed in **Table 2** was added to the plate. Horseradish peroxidase-conjugated secondary antibody (1:2000 in blocking solution) was added after washing the plate two times with washing buffer. The bound secondary antibody was detected using 3,3',5,5'-tetramethylbenzidine (TMB) and stopped with 2 M H₂SO₄. The absorbance of the generated color was measured in an ELISA plate reader at 450 nm.

Dose-dependent binding and effects of heparin and NaCl

The binding of vitronectin to Gpm1p was characterized according to concentration dependency and effects of heparin and NaCl. Concentration-dependent binding of vitronectin to Gpm1p was measured using the method described above with different amounts of added vitronectin (0, 0.25, 0.5 and 1.0 µg). Effect of heparin was measured by pre-incubating vitronectin with increasing amounts of heparin (0, 0.01, 0.1 and 1.0 µg) for 30 min at 37 °C. To determine the effect of divalent cations to the binding of vitronectin to Gpm1p, increasing concentrations of MgCl₂ (0, 0.1, 0.5 and 1.0 mM) was pre-incubated with vitronectin before adding to Gpm1p as described above. The same procedure was used to determine whether the binding was based on ionic strength with the addition of increasing concentrations of NaCl (0, 50, 100, 150, 300 and 600 mM).

Binding of complement proteins to Tef1p

The binding of different complement proteins to immobilized Tef1p was done using the steps in Section 2.2.3.1. but using Factor H, C4BP, C3d, C3dg, iC3b or plasminogen as ligand, and Tef1p was immobilized instead of Gpm1p.

Primary and secondary antibodies used to detect the corresponding bound ligands are listed in **Table 2**.

Binding of plasminogen to Tef1p

Dose-dependent binding of plasminogen to immobilized Tef1p was done using the steps in Section 2.3.1.2 but using plasminogen concentrations of 0, 1.0, 5.0, and 10 µg/ml. To determine if lysine residues are involved in the binding of plasminogen to Tef1p, various concentration of the lysine analog ε-aminocaproic acid (ε-ACA) (0, 0.1, 0.2, 0.4, and 1.0 mM) was first incubated with plasminogen (5.0 µg/ml) for 30 min at 37 °C before adding to immobilized Tef1p. Bound plasminogen was detected by goat plasminogen antiserum followed by HRP-coupled rabbit anti-goat IgG.

2.2.3.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Protein mixtures were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to method of Laemmli [114] using a Biometra system. Protein samples were diluted in loading buffer (0.31 M tris/base pH 6.8, 5% SDS, 50% glycerol, 0.001% bromphenol blue) or denatured by β-mercaptoethanol-containing Roti®-Load 1 (Roth) at 95°C for 5 min. Electrophoresis was performed using 4 % stacking gels (3.0 ml distilled water, 1.25 ml 0.5 M tris/HCl pH 6.8 plus 0.4% SDS, 0.650 ml acrylamide-bisacrylamide 30%, 25 µl 10% ammonium persulfate (APS), 5 µl N,N,N',N'-tetramethylethylenediamine (TEMED) and 8% or 12% running gels (for 12%: 5.25 ml distilled water, 5.25 ml 1.5 M tris/base pH 8.8 plus 0.4% SDS, 5.0 ml acrylamide-bisacrylamide 30%, 50 µl 10% APS, 10 µl TEMED) in running buffer (3% tris/base, 14.4% glycine, 1% SDS).

2.2.3.3. Silver staining

To detect proteins after protein electrophoresis, silver staining was performed. Gels were fixed in 30% acetic acid plus 20% ethanol for 45 min, washed twice with 20% ethanol and sensitized in 0.02% sodium thiosulfate for 2 min. Then the gels were rinsed twice for 1 min in distilled water, stained for 20 min with 0.2% silver nitrate, and rinsed twice in distilled water again. Gels were developed using developer (0.00007% formaldehyde (37%), 3% sodium carbonate, 0.001% sodium thiosulfate), and the reaction was stopped by rinsing with stop solution (2.5% acetic acid, 50% tris/base).

2.2.3.4. Coomassie blue staining

Coomassie staining (Page Blue, Fermentas) was performed according to the manufacturer's instructions.

2.2.3.5. Western blotting

To detect specific proteins after SDS-PAGE, Western blotting was performed. Gels were transferred to a nitrocellulose membrane (Protran), by placing them

in the transfer cassette, and immersing them in transfer buffer (0.045 M tris, 0.039 mM glycine, 20% methanol, 0.1% SDS), and allowing to run at 48 mA for 70 min. Membranes were then blocked in blocking buffer (1% bovine serum albumin [BSA], 4% milk powder, 0.1% Tween 20) for 1 h at room temperature or overnight at 4°C, and incubated with appropriate primary and secondary antibodies (Ab) for 1 h at room temperature, washed with wash buffer (0.05% Tween in phosphate buffered saline (PBS) II (137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.46 mM KH₂PO₄), and developed with enhanced chemiluminescence (ECL)-substrate solution (AppliChem) with a DNR BioImaging System (MF-ChemBIS 3.2).

2.2.3.6. Surface plasmon resonance (SPR)

Plasminogen binding to immobilized Tef1p was analyzed in real time by surface plasmon resonance using Biacore 3000 at 25°C in 150 mM PBS. Tef1p was immobilized on a CMD 500M sensor chip (Xantec) by standard amino coupling chemistry following the manufacturer's protocol. Plasminogen (at concentrations of 0, 100, 200 or 400 nM) was injected at a flow rate of 5 µl/min. The effect of on the binding of plasminogen to Tef1p was also investigated using surface plasmon resonance. Plasminogen was incubated with 0, 0.5, 1.0, or 2.0 nM ε-ACA prior to injection to immobilized Tef1p as above.

2.2.3.7. Protein expression and purification

2.2.3.7.1. Gpm1p

Recombinant Gpm1p was expressed and purified in *Pichia pastoris* using the procedure of Poltermann *et al.* (2007) [61].

2.2.3.7.2. Tef1p

One colony of freshly-grown transformant was inoculated into 10 ml LB with ampicillin (100 µg/ml) overnight at 37 °C with shaking until it reached OD₆₀₀ = 1-2. Two ml of grown culture were then inoculated into 100 ml LB+Amp medium at 37 °C with shaking until OD₆₀₀ = 0.5 (2-3 h). One mM IPTG was added to induce expression for 4-6 hours. Cells were collected by centrifugation at 3000 x g for 10 min at 4 °C.

Disruption, washing and isolation of inclusion bodies

Pellet was resuspended from 100 ml *E. coli* culture in 4 ml Lysis Buffer (50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, and 10 mM imidazole; pH=7.8) with 4 µl Benzonase and 80 µl Lysozyme (10 mg/ml). Sample was frozen in liquid nitrogen and then thawed at 42 °C. This step was repeated twice. Cells were disrupted with sonication (3 x 30 sec.) then centrifuged at high speed for 10 min. at 4 °C. Pellet was

resuspended in 3 ml cold Isolation Buffer (2 M urea, 0.02 M Tris-HCl, 0.5 M NaCl, and 2% Triton X-100; pH=8.0) then centrifuged at high speed for 10 min. at 4 °C. This was repeated twice.

Solubilization and sample preparation

Pellet was resuspended in 5 ml Binding Buffer (0.02 M Tris-HCl, 0.5 M NaCl, 0.005 M imidazole, 6 M guanidine hydrochloride, and 0.001 M β -mercaptoethanol; pH=8.0) then rolled for 30-60 min. at RT. Sample was centrifuged for 15 min. at high speed at 4 °C. Remaining particles were removed by passing the sample through a 0.22 or 0.45 μ m filter.

Preparation of the column

HisTrap HP 1 ml column was washed with 5 ml filtered distilled deionized water at RT using a peristaltic pump at 5 min at 1.0 ml/min. The column was then equilibrated with 5-10 ml Binding Buffer (5-10 min at 1.0 ml/min).

Purification and refolding

Sample was loaded at rate of 0.5 ml/min into the column and then the column with sample was washed with 10 ml Binding Buffer at RT. Then, the column was washed with 10 ml Washing Buffer (0.02 M Tris-HCl, 0.5 M NaCl, 0.02 M imidazole, 6 M urea, and 0.001 β -mercaptoethanol; pH=8.0). After the normal water washing step of Äkta purifier, Tubing A was placed in Washing Buffer and Tubing B in Refolding Buffer (0.02 M Tris-HCl, 0.5 M NaCl, 0.02 M imidazole, and 0.001 M β -mercaptoethanol; pH=8.0). Column was placed in the appropriate Column Position on the Äkta. Loaded protein was refolded in the column by using linear gradient function (100% target for B) for 30 ml (60 min at 0.5 ml/min) then continued washing with 5 ml (10 min at 0.5 ml/min) Refolding Buffer after the gradient came to its endpoint. Tubing A was then placed in Refolding Buffer and Tubing B in Elution Buffer (0.02 M Tris-HCl, 0.5 M NaCl, 0.5 M imidazole, and 0.001 M β -mercaptoethanol; pH=8.0). The system was run with 0% B until UV went down to 0. The refolded protein was then eluted (100% target for B) for 10-20 ml (20-40 min at 0.5 ml/min). Further elution was done for 5 ml more or until UV became near 0. The target for B was brought down to 0% then the column was removed from the Äkta purifier. Eluted protein was desalted using desalting column (Disposable PD-10, GE Healthcare Life Sciences) and concentrated using centrifugal filter units (Millipore). Buffer was exchanged to DPBS during desalting and concentration. Protein concentration of purified Tef1p was determined by NanoDrop, and the purity was analyzed by SDS-PAGE, and silver staining or Western blotting.

2.2.3.8. Generation of protein specific antibodies

Gpm1p mouse monoclonal antibodies were kindly generated by the laboratory of Prof. Dr. Reinhard Wallich (Universitätsklinikum Heidelberg) after providing

them with purified recombinant Gpm1p. Tef1p rabbit antisera were obtained from David's Biotechnologie after submitting purified recombinant Tef1p.

2.2.3.9. Detection of Tef1p on the surface of *C. albicans* cells

The presence of Tef1p on the surface of both yeast and true hyphal forms of *C. albicans* was detected using the generated Tef1p rabbit antiserum. A colony of GFP-expressing *C. albicans* was inoculated in YPD broth at 30 °C overnight. Cells from this culture were used for the subsequent experiments. *C. albicans* true hyphal cells were obtained by incubating yeast cells in YPD broth for 1.5 h at 37 °C. *C. albicans* yeast or hyphal cells (10^4) were inoculated on 14-mm coverslip in DPBS and allowed to adhere for 2 h. After washing twice with DPBS+0.05% Tween 20, *C. albicans* cells were incubated with Tef1p rabbit antiserum for 1 h at RT. Alexa Fluor® 647-conjugated goat anti-rabbit was used as secondary antibody after washing with DPBS+0.05% Tween 20. The coverslips were observed for fluorescence under the laser scanning microscopy (LSM).

2.2.3.10. Biotinylation of proteins

Biotinylated Gpm1p or BSA was generated using the EZ-Link Sulfo-NHS-Biotinylation kit (Pierce) and following the manufacturer's instructions.

2.2.4. Functional Assays

2.2.4.1. Acquisition of vitronectin from serum by *C. albicans*

To determine if *C. albicans* can acquire vitronectin from serum, adsorption assay in combination with Western blotting or flow cytometry was carried out. Yeast cells (1.0×10^5) were washed twice in phosphate-buffered saline (PBS) and then incubated with 10% heat-inactivated normal human serum (hiNHS) for 30 min at 37 °C. Normal human serum was heat-inactivated by incubating at 56 °C for 30 min. For Western blotting, samples were mixed in loading buffer and heated at 99 °C for 10 min with shaking. Then, samples were run in SDS-PAGE using 10% gel. Proteins were transferred to nitrocellulose membrane and blocked in blocking solution (same as above) overnight at 4 °C. The membrane was then incubated in polyclonal rabbit anti-vitronectin antibody (1:1000 in blocking solution) for 1 h at RT. After washing three times (10 min each) in washing buffer (same as above), the membrane was incubated in horseradish peroxidase-conjugated goat anti-rabbit (1:2000 in blocking solution) for 1 h at RT.

For flow cytometry, yeast cells were washed in PBS with 1% BSA before incubating in polyclonal rabbit anti-vitronectin antibody (1:100 in PBS + 1% BSA) for 30 min at RT. After two washes, cells were incubated in Alexa-fluor 647-labeled goat anti-rabbit IgG (1:200) for 30 min at RT. Cells were then washed and examined by flow cytometry. Forward and sideward scatters

were used for the identification of the cells, and fluorescent events of 10,000 cells were counted.

2.2.4.2. Blocking assays

To determine if surface Gpm1p of *C. albicans* plays a role in the acquisition of vitronectin from human serum, blocking assays were done using *Candida* ELISA and flow cytometry. *Candida* ELISA was carried out by coating 1.0×10^5 yeast cells on 96-well microtiter plate with carbonate-bicarbonate coating buffer overnight at 4 °C. After blocking, wells were added with 10% hiNHS pre-incubated with varying amounts of recombinant Gpm1p (0, 0.1, 1.0 and 10.0 µg) at 37 °C for 30 min. The bound vitronectin was detected using the procedure described above. Flow cytometric determination of the amount of vitronectin which bound to the yeast cells was done as above but with the pre-incubation of hiNHS with varying amounts of recombinant Gpm1p.

2.2.4.3. Gpm1p binding to human cells

Determination of the binding of recombinant Gpm1p to both endothelial cells and keratinocytes was done using flow cytometry and laser scanning microscopy (LSM). For the flow cytometry experiment, human cells were collected and washed with PBS+1%BSA twice. Recombinant Gpm1p was added to the cells at increasing amounts (0, 1.25, 2.5 and 5.0 µg) and incubated for 30 min at 37 °C. Bound Gpm1p was detected with rabbit polyclonal anti-Gpm1p antiserum (1:100) followed by Alexa 647-labeled goat anti-rabbit IgG (1:200) and measured using flow cytometer. To know the effect of vitronectin on the binding of Gpm1p to human cells, vitronectin (0.1 µg/µl) was pre-incubated with biotinylated Gpm1p prior to addition to HUVEC and keratinocytes. Bound Gpm1p was quantified using Streptavidin-conjugated Cy5 in flow cytometer.

Laser scanning microscopy was used to view the binding of Gpm1p to human cells. Confluent host cells grown on 14-mm coverslips in a 24-well tissue culture plate were added with 10 mg/ml recombinant Gpm1p in FCS-free DMEM and incubated for one hour at 37 °C with 5% CO₂. After several washes with PBS+1%BSA, rabbit polyclonal anti-Gpm1p antiserum was added at a dilution of 1:100 and incubated for 30 min at RT. Alexa 488-labeled goat anti-rabbit IgG (1:200) was then added to the wells for 30 min at RT. Wells were washed at least twice between the additions of the two antibodies. The coverslips were removed from the wells and viewed using a laser scanning microscope.

2.2.4.4. Detection of presence of vitronectin on human cells

To determine whether HUVEC and keratinocytes endogenously express vitronectin, immunological detection of vitronectin was measured using flow cytometry and laser scanning microscopy. Steps in Section 2.2.4.3. were

followed with the exception of the addition of Gpm1p. To detect vitronectin, rabbit polyclonal anti-vitronectin antiserum was used as primary antibody and Alexa 488-labeled goat anti-rabbit IgG was used as secondary antibody.

2.2.4.5. Colocalization of Gpm1p and vitronectin on surface of human cells

To observe the colocalization of Gpm1p with the vitronectin on the surface of HUVEC and keratinocytes, laser scanning microscopy was used. Gpm1p and vitronectin were detected on the surface of human cells by following the procedure described in Section 2.2.4.3. with the use of monoclonal Gpm1p mouse antibody and polyclonal vitronectin rabbit antiserum. Appropriate secondary antibodies used are listed in **Table 2**. DAPI (4',6-diamidino-2-phenylindole) was also added in the antibody mixture to visualize the nucleus of the human cells.

2.2.4.6. Measurement of host cell-associated *C. albicans* or latex beads

The amount of *C. albicans* cells or latex beads which associated (adhered and endocytosed) with the host cells were measured by flow cytometry and viewed using confocal laser scanning microscopy. The host cells were grown in 24-well tissue culture plate until confluent. Prior to inoculation with *C. albicans* yeast cells, host cells were stained with DiO. After several washes, DiD-stained fungal cells (1.0×10^5) were inoculated and the plate was incubated for 2 hours at 37 °C with 5% CO₂. Host cells were removed from the wells by using Accutase. Host cell-associated *C. albicans* cells were measured by determining the amount of host cells with double positive signal for DiO and DiD after gating using the forward and sideward scatters.

To view and measure the amount of Gpm1p-coated blue fluorescent latex beads (amine-modified polystyrene, Sigma-Aldrich) which became associated with the host cells, indirect immunofluorescence was used. Gpm1p- or biotinylated BSA-coated beads (2.5×10^5) were incubated with confluent host cells on 14-mm diameter coverslips in 24-well tissue culture plate for 45 min at 37 °C with 5% CO₂. Coverslips were viewed using a confocal laser scanning microscope. Each coverslip was divided into four quadrants where a 5x5 tile scan was done per quadrant. The mean intensity of the blue fluorescence per tile scan was obtained using the ZEN 2009 software.

2.2.4.7. Plasmin(ogen) activity assays

2.2.4.7.1. Chromogenic substrate S-2251 degradation assay

Plasmin(ogen) activation was determined by hydrolysis of the PL-specific substrate S-2251 (D-valyl-leucyl-lysine-p-nitroanilide dihydrochloride; Haemochrom Diagnostica). To determine functional activity of recombinant Tef1p, 10 µg/ml plasminogen was immobilized onto a microtiter plate overnight, blocked with 1% bovine serum albumin in DPBS, and plasminogen

was activated using 0.08 µg/ml tPA together with the chromogenic substrate S-2251 dissolved in reaction buffer (64 mM tris, 350 mM NaCl, 0.01% triton-X; pH 7.5). Plasmin activity was recorded at 1 h intervals at 405 nm (SpektraMax 190; Molecular Devices).

2.2.4.7.2. Fibrinogen and vitronectin degradation assay

Activity of Tef1p-bound plasminogen was further analyzed by degradation of fibrinogen or vitronectin. Ten µg/ml Tef1p was immobilized on microtiter plate wells then 20 µg/ml plasminogen, 1 µg/ml tPA, and 20 µg/ml fibrinogen or vitronectin were incubated for 2 h at 37°C. Samples were reduced with RotiLoad separated by SDS-PAGE, and transferred to a nitrocellulose membrane. Fibrinogen degradation fragments were identified using anti-fibrinogen rabbit antibody (Calbiochem) and secondary goat rabbit antibody, while vitronectin degradation products were detected by vitronectin rabbit antiserum and HRP-coupled goat rabbit antibody.

2.2.4.7.3. C3b degradation assay

To characterize the activity of Tef1p-bound plasminogen activity of C3b degradation, 20 µg/ml Tef1p was immobilized on microtiter plate wells, then 20 µg/ml plasminogen was added and incubated for one h at 37°C. Then, 1 µg/ml tPA, and 10 µg/ml C3b was added after extensive washing. Mixtures were incubated for 4 h at 37°C wherein 100 µl samples were collected every hour. Samples were reduced with Roti-Load for 5 min at 95°C, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. C3b and their cleavage products were detected with anti-C3-Fab-HRP (1:1000).

2.2.4.8. Binding of complement proteins to *C. albicans* cells

C. albicans SC5314, *tef1* null mutant and reintegration strains were incubated with Factor H, C4BP, C3d or plasminogen (10 µg/ml) for 1 h at 37°C. After washing twice with DPBS+1% BSA, bound proteins were detected using appropriate primary and fluorescent dye-conjugated secondary antibodies (**Table 2**). Antibodies were incubated with *C. albicans* cells for 30 min at RT after fixation with 3% paraformaldehyde. Amount of complement proteins which bound to *C. albicans* cells were quantified using flow cytometry.

2.2.4.9. Reactive oxygen species (ROS) assay

The ROS assay was performed by Pedro Miramón (Department of Microbial Pathogenicity Mechanisms, Leibniz Institute for Natural Product Research, HKI). Briefly, neutrophils were seeded at a rate of 5×10^4 cells in each well of a microtiter plate which was coated with 0.05% albumin. Cells were incubated at 37 °C, 5% CO₂, for one hour in phenol red-free RPMI with 4% fetal bovine serum. Fifty µl of *C. albicans* cell suspension with a concentration of 1×10^6 (MOI=1) was added to each microtiter plate well. Fifty µl of luminol solution (in 10 ml solution: 20 µl 0.1 M luminol in DMSO, 80 µl horseradish peroxidase,

and 9900 µl RPMI without phenol red and FCS) were then added to the mixture in each well. Luminescence was immediately measured and monitored for one h with a reading every 2.5 min in a fluorescence reader (Tecan).

2.2.5. Maintenance and cultivation of human cell lines

HUVEC cells were maintained in DMEM while HaCaT cells were maintained in RPMI 1640. Media were supplemented with 10% fetal calf serum (FCS), 1% ultraglutamine 1, and 0.055% gentamicin sulfate. Cells were incubated at 37 °C with 5% CO₂. Human cells were passaged every 3 days and only cells with less than 30 passages were used in the experiments.

2.2.6. Statistical analyses

Standard deviations and significant differences between test groups were analyzed by either t-test or analysis of variance (ANOVA) using the software GraphPad Prism 6 (GraphPad Software, Inc.)

3. RESULTS

3.1. Phosphoglycerate mutase (Gpm1p)

3.1.1. *Candida albicans* Gpm1p binds to human cells

The *Candida albicans* surface protein Gpm1p binds three human innate immune proteins, i.e., Factor H, Factor H-like protein 1 and plasminogen. It was therefore hypothesized that fungal Gpm1p binds additional human proteins and has additional functions, such as binding to human cells. To this end, binding of *Candida* Gpm1p to human cells was analyzed using endothelial cells (HUVEC), keratinocytes (HaCaT), and the pre-monocytic cell line U937. Recombinant Gpm1p was added to the human cells and after washing, bound Gpm1p was detected with Gpm1p antiserum and quantified by flow cytometry. Gpm1p bound to HUVEC and HaCaT but not to U937. More Gpm1p bound to HUVEC (MFI=1515) than to HaCaT cells (MFI=768) (**Figure 4A**). In addition, Gpm1p binding to the two human cell lines was visualized by confocal laser scanning microscopy (LSM). Gpm1p bound to the surface of HUVEC and HaCaT as revealed by the green fluorescence (**Figure 4B**). Bound Gpm1p was localized to the edge of the individual cells in a continuous pattern. Taken together, *C. albicans* protein Gpm1p binds to human endothelial cells and keratinocytes, but not to monocytes.

3.1.2. Deletion of GPM1 decreases association of *C. albicans* to human endothelial cells

The role of Gpm1p as a *C. albicans* surface protein mediating interaction with human cell e.g. HUVEC was analyzed using *C. albicans* SC5314 (wild type), GPM1 heterozygous deletion mutant (*gpm1Δ*), GPM1 homozygous deletion mutant (*gpm1Δ/Δ*), and GPM1 reintegration strain (*gpm1Δ/Δ::GPM1*). The various *C. albicans* strains were incubated together with HUVEC cells and after washing, the fraction of associated (adhered and/or endocytosed) yeast cells was quantified using flow cytometry. GPM1 homozygous deletion mutant cells bound less to HUVEC (more than 60% decrease) compared to wild type strain (**Figure 5**). The number of cells of heterozygous mutant and reintegration strain which bound to HUVEC was similar as the wild type. These results show that Gpm1p mediates adhesion of *C. albicans* with human endothelial cells.

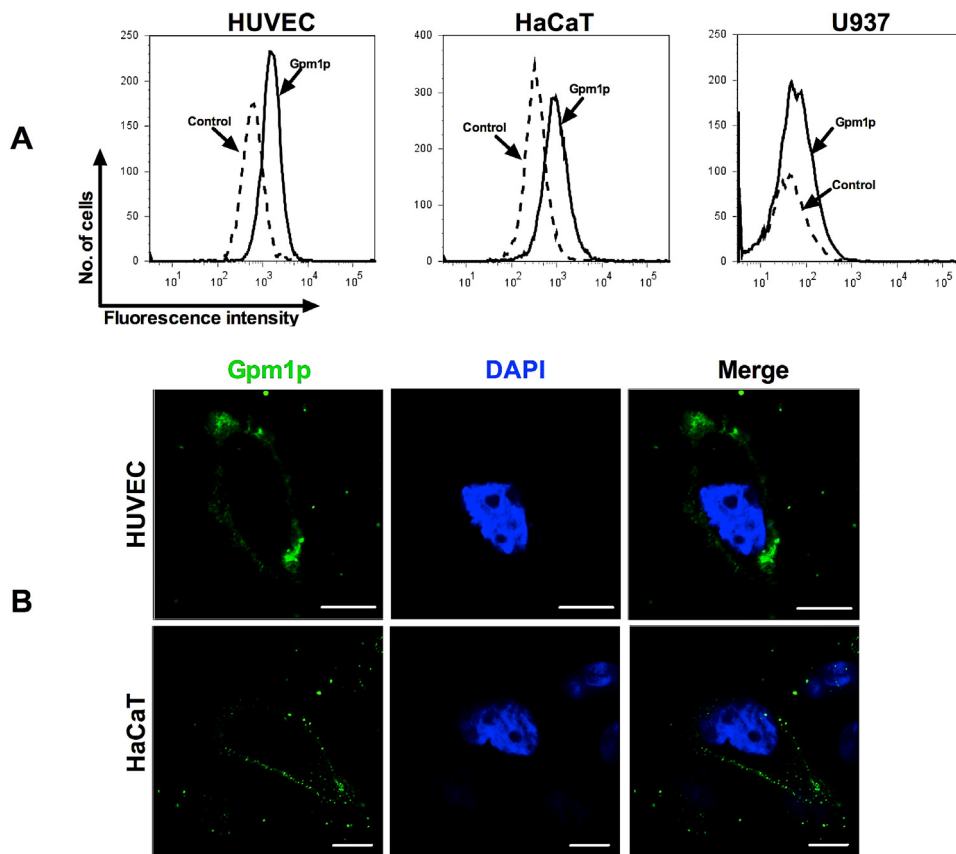


FIGURE 4. *Candida albicans* Gpm1p binds to human cells

(A) Gpm1p was added to HUVEC, HaCaT or U937. After incubation and subsequent washing, bound Gpm1p was detected in flow cytometry using rabbit Gpm1p antiserum followed by Alexa Fluor® 647 goat anti-rabbit IgG. Human cells without Gpm1p were used as control. Histograms are representatives of 3 independent experiments. (B) Binding of Gpm1p to HUVEC and HaCaT was confirmed by laser scanning microscopy. HUVEC (top panel) or HaCaT (bottom panel) was incubated with Gpm1p, fixed with paraformaldehyde and after washing, bound Gpm1p was detected with rabbit Gpm1p antiserum followed by Alexa Fluor® 488 goat anti-rabbit IgG (green). DNA of the cells was stained with DAPI (blue). Scale bar = 10 μ m.

3.1.3. Association of Gpm1p-coated latex beads to endothelial cells

To define a direct role of Gpm1p in fungal interaction with human endothelial cells, and to eliminate the effect of other fungal surface proteins, attachment of Gpm1p-coated latex beads to HUVEC was assessed. Gpm1p was coated to blue fluorescent latex beads and the Gpm1p-coated latex beads were incubated with HUVEC cells. Bound beads were quantified by measuring their blue fluorescence from a specific area of HUVEC by confocal laser scanning microscopy. More Gpm1p-coated latex beads bound to HUVEC ($\text{MFI}/\mu\text{m}^2=2.5$) than with latex beads coated with BSA ($\text{MFI}/\mu\text{m}^2=1.6$) (**Figure 6**). Thus, Gpm1p is an adhesion protein of the fungal pathogen *C. albicans* that contributes to the adherence to human endothelial cells.

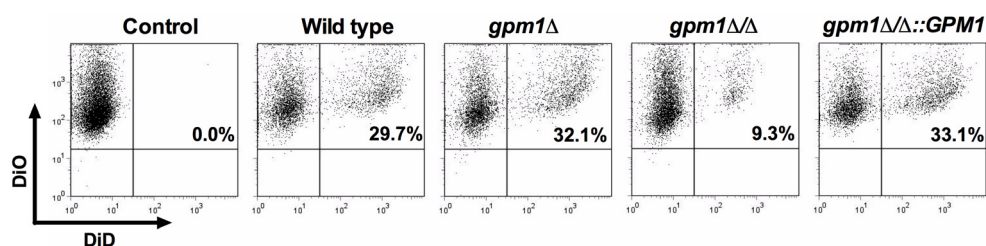


FIGURE 5. *GPM1* knock-out strain decreases association to endothelial cells.

Yeast cells of *C. albicans* SC5314 (wild type), *gpm1*Δ, *gpm1*Δ/Δ or *gpm1*Δ/Δ::GPM1 was labelled with DiD and incubated with DiO-labelled HUVEC for 120 min at 37 °C with 5% CO₂. After washing and detachment, HUVEC with associated (adhered and/or endocytosed) *C. albicans* was identified in flow cytometry as double-positive cells (DiO+, DiD+) by the change in side scatter. HUVEC alone detected as single-positive cells (DiO+, DiD-) were used as control.

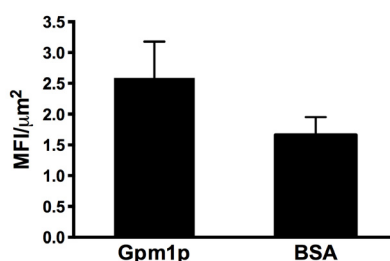


FIGURE 6. Association of latex beads with Gpm1p to endothelial cells

Blue fluorescent latex beads were coated with Gpm1p or biotinylated BSA and incubated with HUVEC for 45 min at 37 °C with 5% CO₂. HUVEC-associated latex beads were quantified in LSM where blue fluorescence was measured per μm² under the oil immersion objective lens using the ZEN 2009 software. Data are mean ± SD (error bars) of three experiments.

3.1.4. *Candida* Gpm1p binds human extracellular matrix protein vitronectin

In order to identify host proteins or ligands for *Candida* Gpm1p that are expressed on the surface of human cells, it was hypothesized that *Candida* Gpm1p would interact with human extracellular matrix components (ECM). Therefore, binding of various human ECM proteins to recombinant Gpm1p was determined. Immobilized *Candida* Gpm1p was bound by human vitronectin and fibronectin, but not by laminin, fibrinogen, collagen I, collagen III, nor collagen IV (**Figure 7A**). Since vitronectin showed the most binding to Gpm1p, the interaction of the two proteins was analyzed in more detail.

First, dose-dependent binding of vitronectin to immobilized Gpm1p was analyzed using ELISA. *Candida* Gpm1p was immobilized and vitronectin (in increasing amounts) was added. Bound vitronectin was detected by specific vitronectin antiserum. Vitronectin bound to Gpm1p, and binding was dose-dependent (**Figure 7B**), indicating the specificity of the interaction. In addition, it was determined if heparin-binding regions are involved in the interaction

between Gpm1p and vitronectin. To this end, the effect of heparin on vitronectin binding to immobilized Gpm1p was determined. First, heparin was added to vitronectin and then the heparin-vitronectin complex was added to immobilized Gpm1p. After washing, bound vitronectin was detected in ELISA. Heparin inhibited vitronectin binding to immobilized Gpm1p and the effect was dose dependent. An approximately 20% decrease in the binding of vitronectin to Gpm1p was measured when 0.1 μg heparin was used. Increasing the concentration of heparin 10 times (*i.e.*, 1.0 μg) inhibited vitronectin binding to Gpm1p by almost 60% (**Figure 7C**).

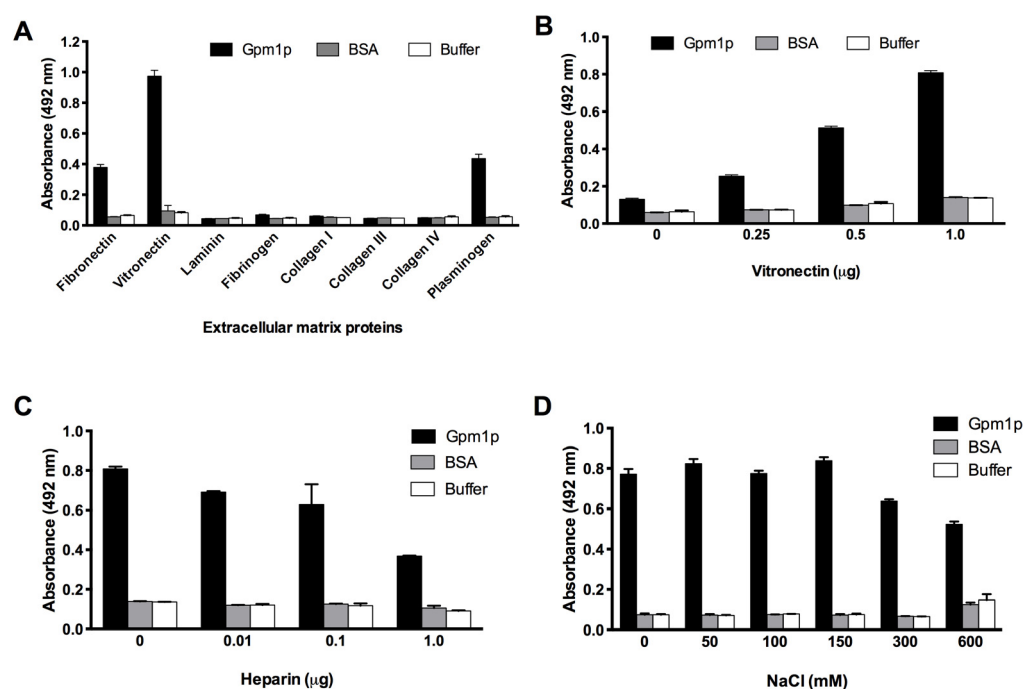


FIGURE 7. Gpm1p binds to human vitronectin

(A) Vitronectin and fibronectin bind to Gpm1p. Binding of extracellular matrix (ECM) proteins to Gpm1p was determined in ELISA. Gpm1p was immobilized on a microtiter plate and added with fibronectin, vitronectin, laminin, fibrinogen, collagen I, collagen III, or collagen IV. Bound ligand was detected with antiserum specific to each ECM protein followed by HRP-conjugated polyclonal IgG specific to the antiserum. Binding of plasminogen to Gpm1p was used as control. (B) Vitronectin dose-dependently binds to immobilized Gpm1p. Gpm1p was immobilized on a microtiter plate, vitronectin was added in indicated amounts, and bound vitronectin was detected by rabbit vitronectin antibody, followed by HRP-conjugated polyclonal goat anti-rabbit IgG. (C) Heparin inhibits the binding of vitronectin to Gpm1p. Vitronectin and heparin (in indicated amounts) were pre-incubated before adding to immobilized Gpm1p. Bound vitronectin was detected as in (B). (D) Interaction of vitronectin with Gpm1p is affected by ionic strength. Vitronectin was pre-incubated with NaCl (in indicated final concentrations) and added to immobilized Gpm1p. Bound vitronectin was detected as in (B). BSA and buffer were used as protein negative and antibody controls, respectively. Data are mean \pm SD (error bars) of three independent experiments.

In addition, the effect of NaCl on the Gpm1p-vitronectin interaction was assessed to determine if the interaction is ionic strength-dependent. NaCl at the physiological level of 150 mM did not affect vitronectin binding. However, at higher levels of 300 and 600 mM, NaCl reduced the binding of vitronectin to Gpm1p by approximately 24% and 38%, respectively, compared to the physiological concentration (**Figure 7D**). Thus, vitronectin binds to Gpm1p via heparin-binding sites and the interaction is affected by ionic conditions.

3.1.5. Vitronectin blocks binding of Gpm1p to human cells

As *C. albicans* Gpm1p binds to human vitronectin and also to human cells, it was hypothesized that vitronectin is one of the major surface ligands for *Candida* Gpm1p on the surface of human cells. To test this, an assay was done to know if vitronectin when added to fluid phase Gpm1p influences or blocks Gpm1p binding to human cells. To this end, vitronectin was added to Gpm1p in solution, then the mixture was added to HUVEC or HaCaT cells and after washing bound Gpm1p was assayed with appropriate antiserum. Vitronectin added to Gpm1p reduced Gpm1p binding to both endothelial cells and keratinocytes (**Figure 8**). Vitronectin used at 10 μ g inhibited Gpm1p binding to HUVEC by 91% and keratinocytes by 75%. Thus, vitronectin complexed to Gpm1p, blocks binding of the fungal protein to human cells.

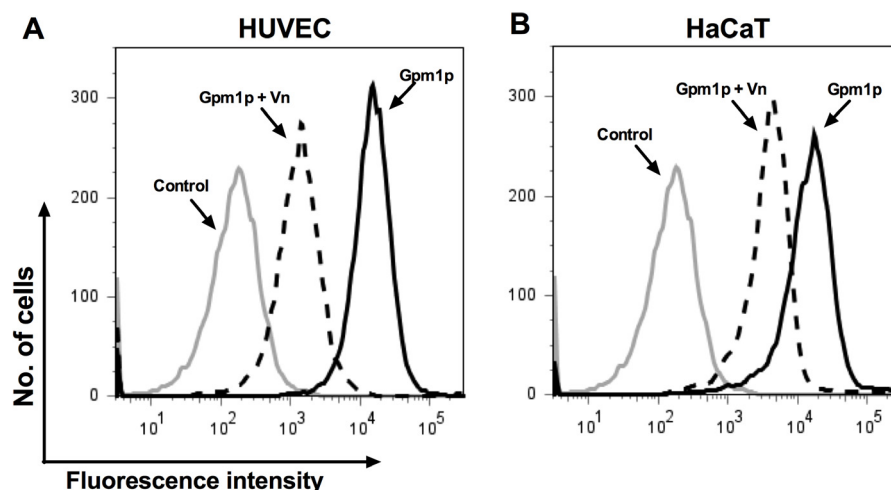


FIGURE 8. Vitronectin blocks binding of Gpm1p to human cells

Biotinylated Gpm1p (10 μ g) was pre-incubated with or without vitronectin (10 μ g) before adding to HUVEC (A) or HaCaT (B). After washing, bound Gpm1p was detected using streptavidin-conjugated Cy5 in flow cytometry. Gpm1p when complexed with vitronectin (black broken line) showed less binding to human cells than Gpm1p alone (black solid line). Human cells without Gpm1p and/or vitronectin was used as control (gray solid line).

3.1.6. Vitronectin is present on the surface of human endothelial cells and keratinocytes

Vitronectin is an adhesion cell surface protein of human cells and a regulator of the complement system [11,100]. Based on the interaction and the blocking effects, it was hypothesized that vitronectin is exposed at the cell surface and acts as a ligand for *C. albicans* Gpm1p. Therefore, vitronectin expression and surface localization on human cells, which were cultivated in serum-free medium, was analyzed by flow cytometry using monoclonal vitronectin antibody. Significant amount of vitronectin was detected on the surface of both HUVEC and HaCaT with an MFI of 2,153 and 819, respectively (**Figure 9A**).

In addition, the surface distribution of vitronectin was visualized by confocal microscopy. Vitronectin on cell surface was labelled with polyclonal rabbit anti-vitronectin which was then labelled with Alexa 488-conjugated anti-rabbit secondary antibody. Human cell membrane was stained with Texas Red-conjugated wheat germ agglutinin (WGA). Vitronectin (green) was evenly distributed on the surface of both HUVEC and keratinocytes which appeared in red (**Figure 9B**). Colocalization of both vitronectin and human cell membrane appears in yellow. Taken together, vitronectin is expressed on the surface of both HUVEC and HaCaT cells.

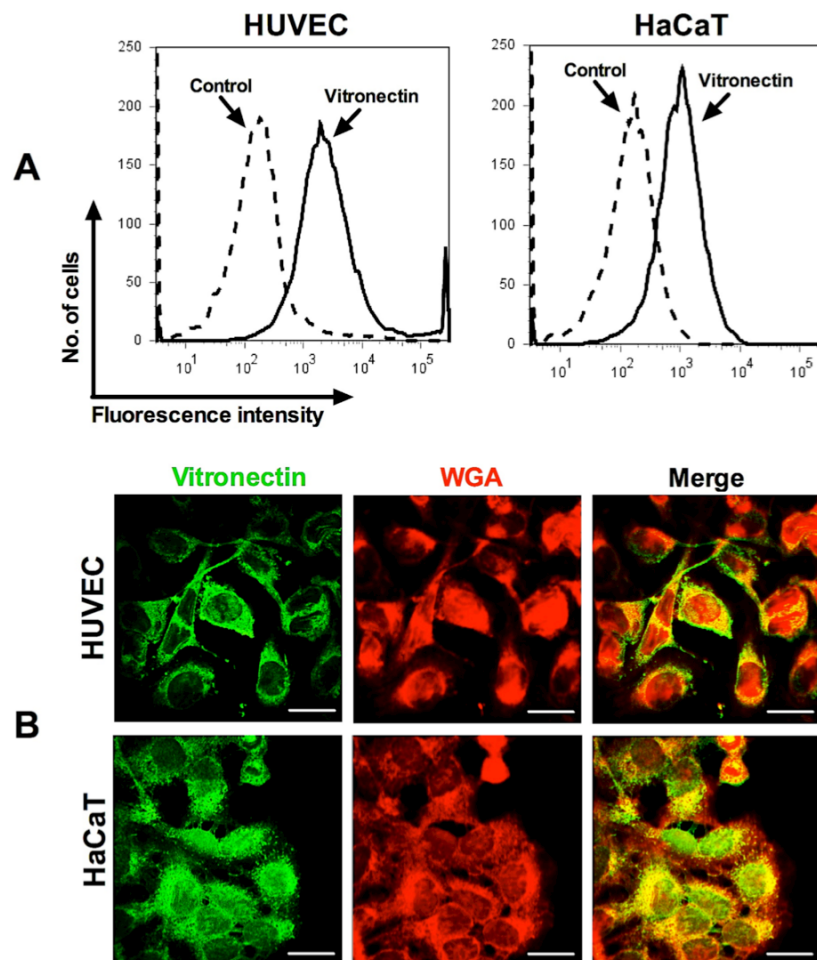


FIGURE 9. Vitronectin is present on the surface of endothelial cells and keratinocytes. HUVEC or HaCaT cells were grown in DMEM or RPMI, respectively, until confluent. After washing, human cells were grown in appropriate medium without serum for 24 h. (A) Human cells were detached from culture flask and vitronectin expressed on the surface of the cells was detected by rabbit vitronectin antibody followed by Alexa Fluor® 647-conjugated goat anti-rabbit IgG as secondary antibody. Fluorescence intensity of detected vitronectin is represented by solid line. Fluorescence intensity of human cells added only with secondary antibody was used as control represented by broken line. (B) Expression of vitronectin by human cells was visually confirmed using LSM. Human cells were grown on coverslip in 24-well plate with appropriate medium until confluent. After washing, human cells were grown in serum-free medium without for 24 h. Cells were fixed with 3% paraformaldehyde and after extensive washing, vitronectin expressed on the surface of the cells was detected by rabbit vitronectin antibody followed by Alexa Fluor® 488-conjugated goat anti-rabbit IgG (green). Human cell membrane was also labelled with Texas red-conjugated wheat germ agglutinin (red). Scale bar = 20 μ m.

3.1.7. *Candida* Gpm1p and vitronectin colocalize at the surface of human cells

In order to prove that vitronectin is the major ligand for *Candida* Gpm1p on the surface of HUVEC and HaCaT, it was determined if Gpm1p and vitronectin colocalize at the same sites on human cells using LSM. To this end, Gpm1p was bound to the surface of HUVEC or HaCaT cells cultivated in serum-free medium. Bound Gpm1p was detected with monoclonal Gpm1p antibody while bound vitronectin was detected with vitronectin antiserum. Gpm1p (green) and vitronectin (red) were detected on the surface of human cells and a merge of the two images revealed that the two proteins colocalize at the same sites (yellow) (**Figure 10**). This indicates that the fungal Gpm1p protein specifically binds to vitronectin on the surface of both human endothelial cells and keratinocytes.

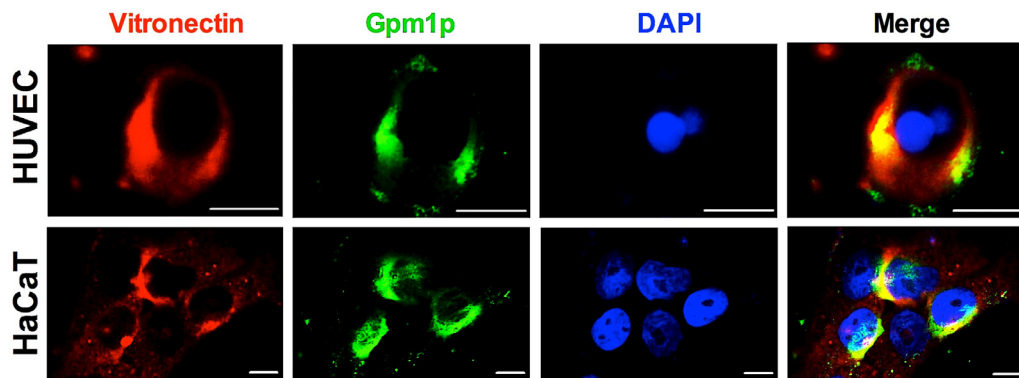


FIGURE 10. Gpm1p and vitronectin colocalize at the surface of human cells

Gpm1p (green) is shown to colocalize with vitronectin on the surface of human cells (red) using LSM. HUVEC or HaCaT cells in serum-free medium was added with recombinant Gpm1p. Human cells with bound Gpm1p were fixed with 3% paraformaldehyde. Gpm1p was detected using monoclonal mouse Gpm1p antibody followed by Alexa Fluor® 488-conjugated goat anti-mouse IgG. Vitronectin expressed on the surface of the cells was detected by polyclonal rabbit vitronectin antibody followed by Alexa Fluor® 647-conjugated goat anti-rabbit IgG. DNA of human cells was stained with DAPI. Scale bar = 10 μ m.

3.1.8. *Candida albicans* uses Gpm1p at the surface to acquire vitronectin from human serum

Vitronectin as an adhesion protein and ECM component is localized at the surface of human cells [12]. However, it is also secreted and thereby present in soluble form in human plasma at a concentration of ca. 200-400 µg/ml [11]. It was therefore asked whether soluble vitronectin in human serum also binds the surface-expressed Gpm1p of *C. albicans*. Binding of vitronectin from serum to intact *C. albicans* cells was first analyzed prior to investigating the specific binding of vitronectin to Gpm1p on *C. albicans* cell surface. To this end, yeast cells were incubated in normal human serum (NHS) and cells were lysed after washing. The cell lysate was then separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Bound vitronectin was identified in Western blot as 65- and 75-kDa bands (**Figure 11A**). Thus, vitronectin derived from human serum binds to the surface of *C. albicans*. In order to prove that Gpm1p on the fungal surface binds soluble Vitronectin, a blocking assay was done wherein Gpm1p was first added to hiNHS in order to complex soluble vitronectin, then *C. albicans* cells were incubated in this Gpm1p-treated hiNHS. After washing, bound vitronectin was detected by a whole-cell *C. albicans* ELISA or by flow cytometry. Less Vitronectin bound to *C. albicans* yeast cells in whole cell ELISA when the yeast cells were incubated in Gpm1p-treated hiNHS. The blocking effect was dose-dependent. Ten µg Gpm1p were able to effect a 25% decrease in the amount of Vitronectin which bound to *C. albicans* cells (**Figure 11B**). This blocking effect was also confirmed in flow cytometry where *C. albicans* yeast cells were incubated in Gpm1p-treated hiNHS and bound vitronectin was detected by polyclonal rabbit anti-Vitronectin and Alexa 647-conjugated anti-rabbit secondary antibody. As expected, a decrease in the amount of Vitronectin binding to intact yeast cells was measured in samples incubated in Gpm1p-treated hiNHS (**Figure 11C**). An approximately 60% decrease in the bound vitronectin was measured when 10 µg of Gpm1p was pre-added to 10% hiNHS. Taken together, Gpm1p complexes vitronectin from human serum which then blocks binding of the serum protein to *C. albicans*, implying that Gpm1p is the ligand for vitronectin on the surface of *C. albicans*.

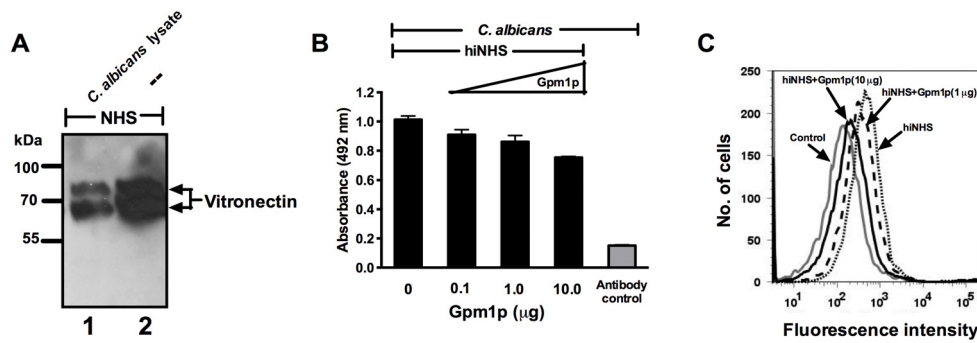


FIGURE 11. *C. albicans* acquires vitronectin from human serum using Gpm1p

(A) *C. albicans* binds vitronectin from normal human serum (NHS). *C. albicans* yeast cells were incubated in 10% NHS for 30 min. Following lysis, cell lysate (Lane 1) and NHS (Lane 2) was loaded on 10% SDS-PAGE gel, transferred onto a nitrocellulose membrane, and vitronectin was detected in Western blot using rabbit vitronectin antibody followed by HRP-conjugated goat anti-rabbit IgG. Vitronectin was identified as the 75- and 65-kDa bands. The migration of the molecular weight protein standards is indicated. (B) Gpm1p decreases the amount of vitronectin which binds to *C. albicans*. The amount of vitronectin from 10% heat-inactivated NHS (hiNHS) with or without Gpm1p (in indicated amounts) which bound to immobilized *C. albicans* was measured in whole-cell ELISA. Bound vitronectin was detected by HRP-conjugated goat anti-rabbit IgG. *C. albicans* cells without hiNHS and Gpm1p were used as antibody control. Data are mean \pm SD (error bars) of three independent experiments. (C) The inhibition of the binding of vitronectin (from hiNHS) to *C. albicans* by Gpm1p was also confirmed in flow cytometry. *C. albicans* yeast cells were incubated in hiNHS with or without Gpm1p (0 μ g, dotted line; 1 μ g, broken line; 10 μ g, black solid line). Bound vitronectin was detected by the same primary antibody as in B but with Alexa Fluor® 647-conjugated goat anti-rabbit IgG as secondary antibody. *C. albicans* cells without hiNHS and Gpm1p were used as control. Histograms are representatives of three independent experiments.

3.2. Translation elongation factor 1-alpha (Tef1p)

3.2.1. Cloning and recombinant expression of *C. albicans* Tef1p

Screening for Factor H-binding proteins of *C. albicans* led to the identification of translation elongation factor 1-alpha (Tef1p) (data not shown). To confirm and characterize the binding of *Candida* Tef1p to the human plasma protein Factor H, a recombinant Tef1p protein was produced using *E. coli*. The *TEF1* gene (orf19.1435) was amplified from the genomic DNA isolated from *C. albicans* SC5314 and subcloned into the pET101 expression vector producing pET101-CaTef1. The plasmid pET101-CaTef1 was designed to have histidine tag at the C-terminus of *TEF1* for purification of the recombinant protein later on. After confirmation of the right insertion of the gene into the vector by sequencing, pET101-CaTef1 was transformed into the *E. coli* expression strain BL21 Star (DE3). Expression of the recombinant Tef1p was induced by IPTG and expression rate was monitored for 6 hours. After cultivation, *E. coli* cells were lysed and lysate supernatant was collected and separated in SDS-PAGE. The presence of Tef1p in the cell lysate supernatant was detected in Western blot using monoclonal penta-His antibody. A 55-kDa band was identified in the lysate supernatant of IPTG-induced culture (**Figure 12A**) but not in non-induced culture (**Figure 12B**). Expression of Tef1p started after one hour of incubation (**Figure 12A, Lane 2**). In addition, the pellet from IPTG-induced cell lysate was also collected and analyzed for the presence of Tef1p as described above. A more prominent Tef1p band was detected in the pellet (**Figure 9B, Lanes 2 and 4**) compared to the supernatant (**Figure 9B, Lanes 1 and 3**), which implied that the recombinant protein was insoluble.

To solubilize the insoluble recombinant Tef1p, a protocol for purification and refolding of His-tagged insoluble recombinant protein produced in *E. coli* from Amersham Biosciences (citation) was used together with the guidelines in the user manual of the Champion pET Directional TOPO Expression Kits by Invitrogen. The procedure included the solubilization of the protein obtained in the pellet of the cell lysate, denaturation, purification by running through Äkta purification system via the His-tag, in-column refolding and elution (**Figure 12D**). The elute fractions were pooled, desalted and concentrated by ultracentrifugation. The wash fractions were also collected during desalting to make sure that no proteins were washed away during the washing process. The concentrated Tef1p sample showed a single prominent band in both silver stained gel and Western blot with molecular mass of 55 kDa (**Figure 12E**).

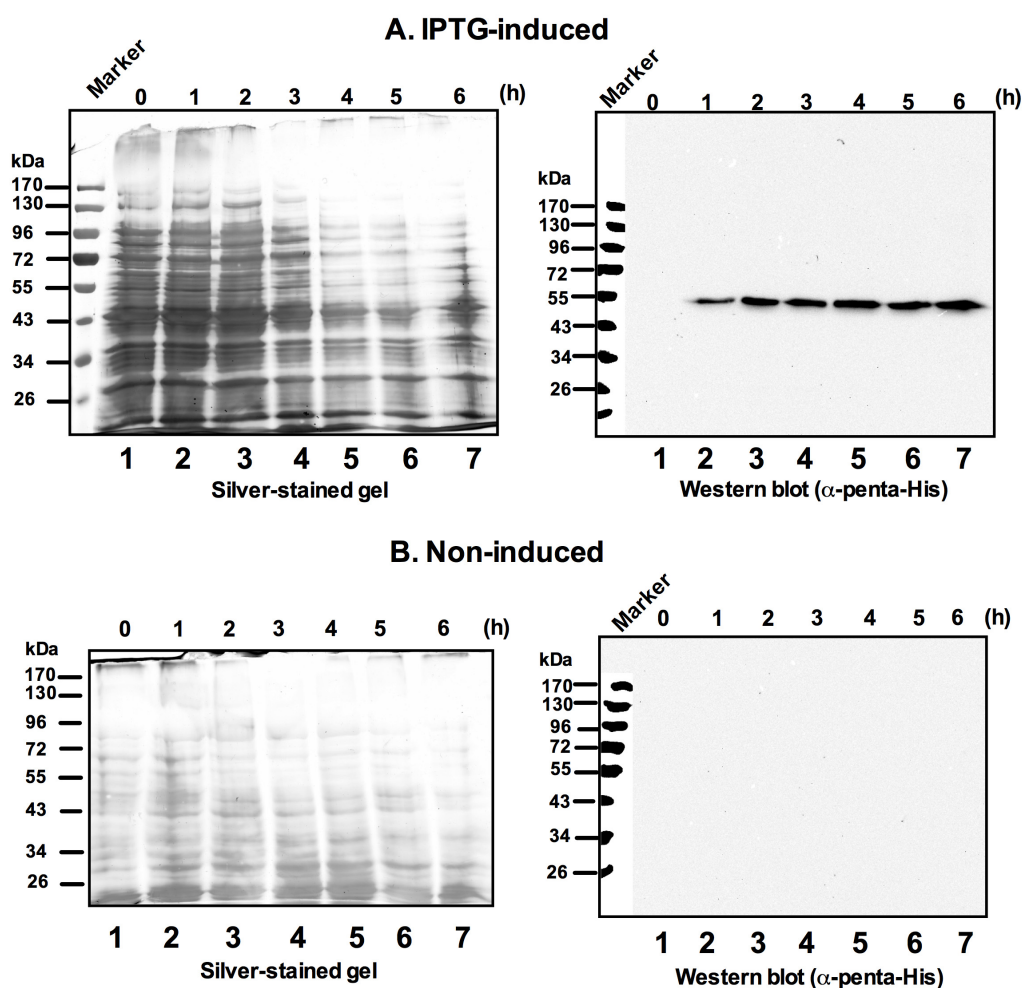


FIGURE 12. Expression and purification of recombinant Tef1p

Test expression of His-tagged Tef1p in the BL21 Star™(DE3) *E. coli* strain harboring the pET101-CaTef1 plasmid was done by incubating cells in LB broth with (A) or without IPTG (1 mM) (B) for 6 h at 37 °C. Ampicillin (100 µg/ml) was added in the medium as selection marker. Culture supernatants were taken every hour and were loaded on 10% SDS-PAGE gels. Gels were silver stained or transferred onto a nitrocellulose membrane for Tef1p detection in Western blot using monoclonal anti-penta-His IgG.

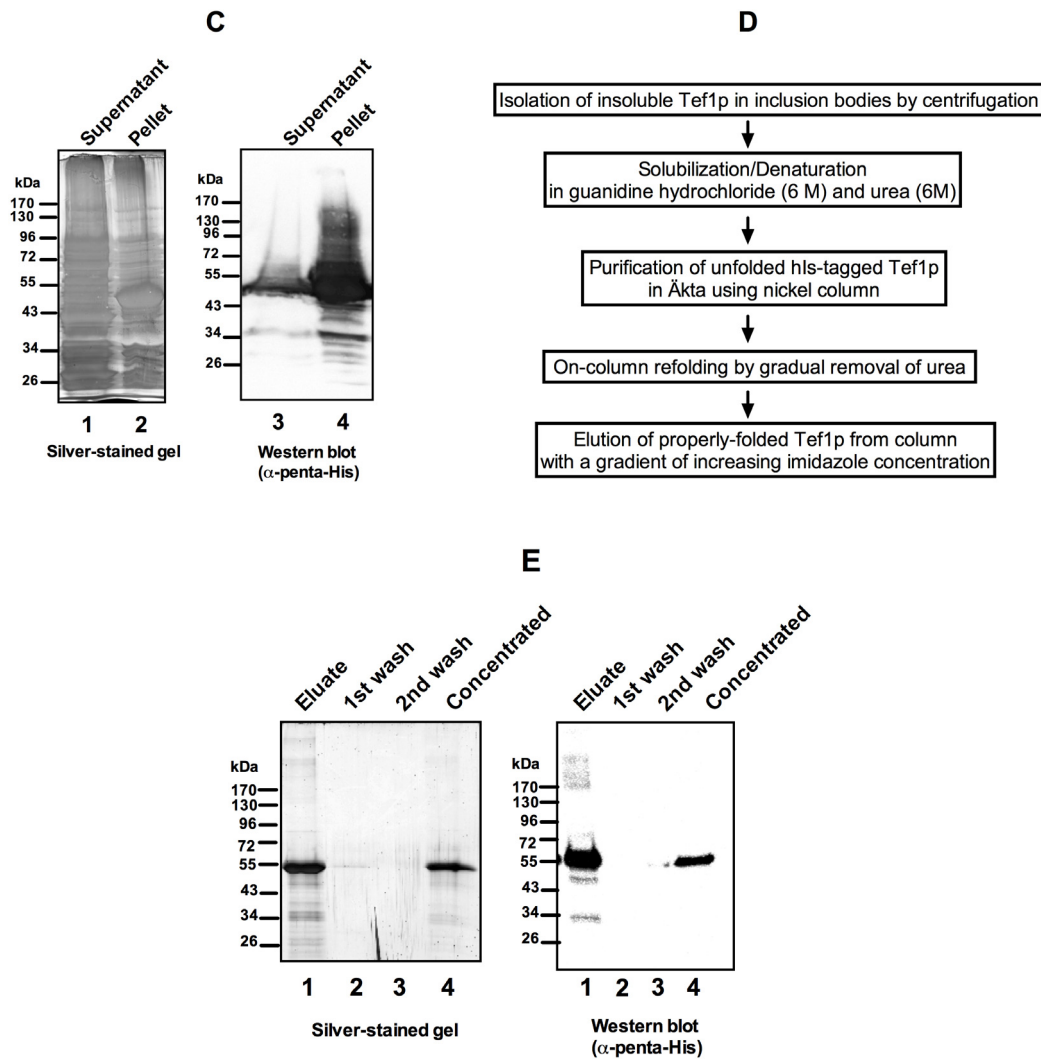


FIGURE 12. Expression and purification of recombinant Tef1p (continued)

(E) Tef1p was eluted from Äkta protein purification system (Lanes 1 and 3), desalted and washed twice with DPBS (Lanes 2-3 and 4-5), and concentrated in ultracentrifuge filters (Lanes 4 and 6). Samples were taken from each step and loaded on 10% SDS-PAGE gel for silver staining (Lanes 1-4) or Western blotting using monoclonal anti-penta-His IgG to detect his-tagged Tef1p. The migration of the molecular weight protein standards is indicated. (C) Recombinant Tef1p is produced as insoluble inclusion bodies in *E. coli* cell lysate pellet. *E. coli* cells were cultured in IPTG-induced LB broth with ampicillin for 4 h at 37 °C. After cell lysis and subsequent centrifugation, supernatant (Lanes 1 and 3) and pellet (Lanes 2 and 4) were collected and loaded on 10% SDS-PAGE gel. Gel was silver-stained (Lanes 1 and 2) or transferred onto nitrocellulose membrane for Tef1p detection in Western blot using monoclonal anti-penta-His IgG (Lanes 3 and 4). (D) Schematic diagram of the workflow for isolation, denaturation, purification, folding and elution of recombinant Tef1p.

3.2.2. *Candida* Tef1p binds human complement proteins

To confirm that *Candida* Tef1p binds to Factor H, an enzyme-linked immunosorbent assay (ELISA) was used. Tef1p was immobilized and Factor H was added in increasing amounts. Bound Factor H was detected by anti-Factor H goat polyclonal antiserum. Factor H bound to immobilized Tef1p and binding was dose-dependent (**Figure 13A**).

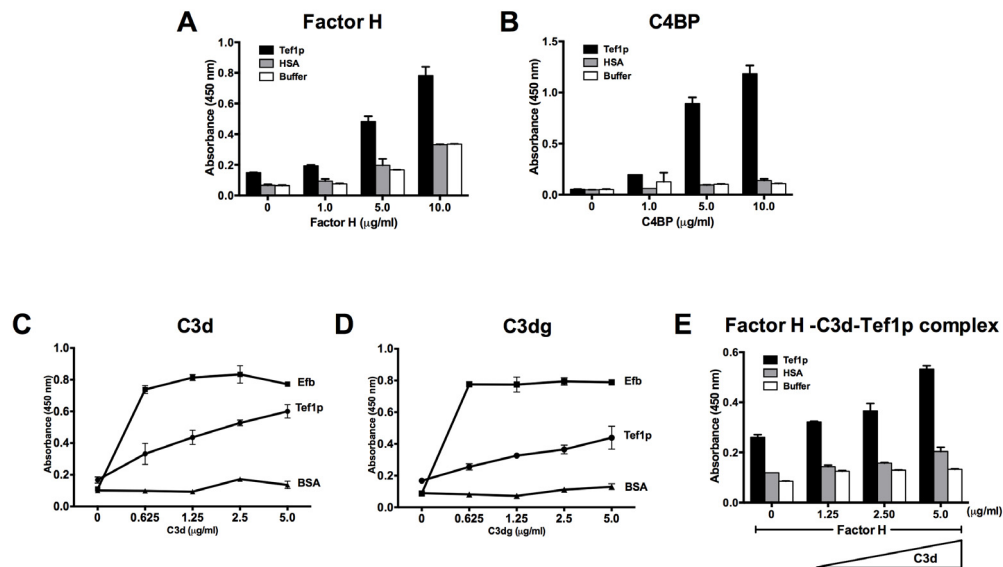


FIGURE 13. Components of the human complement system bind to Tef1p

Binding of components of the human complement system to Tef1p was assayed in ELISA. Tef1p was immobilized on microtiter plate. Factor H (A), C4BP (B), C3d (C), or C3dg (D) was added at indicated concentrations. After washing, bound ligand was quantified using ligand-specific antiserum: goat Factor H antiserum for A, rabbit C4BP antiserum for B, and rabbit C3d antiserum for C and D. The primary antibody was followed by HRP-conjugated rabbit anti-goat IgG (for A) or goat anti-rabbit IgG (for B, C, and D). (E) Factor H binds to Tef1p-bound C3d forming a tripartite complex. Tef1p was immobilized on microtiter plate and added with C3d at indicated concentrations. After washing, Factor H was added and bound Factor H was detected by goat Factor H antiserum followed by HRP-conjugated rabbit anti-goat IgG. The staphylococcal Efb was used as positive control for C3d (C) and C3dg (D). HSA was used as negative control for A, B and E while BSA was used for C and D. Buffer was used as antibody control. Data are mean \pm SD (error bars) of three independent experiments.

To test if *Candida* Tef1p also binds the human complement regulator C4BP, Tef1p was immobilized and C4BP was added in increasing amounts. Bound C4BP was detected in ELISA by anti-C4BP rabbit polyclonal antiserum. C4BP bound to immobilized Tef1p and binding was dose dependent (**Figure 13B**). C3b degradation product C3d was shown to bind to the surface of *C. albicans* and was speculated to help the fungus evade phagocytosis by mediating the clumping of hyphal cells [115]. To determine whether Tef1p interacts with C3d

and C3dg, an ELISA was performed where Tef1p was immobilized and C3d or C3dg binding was studied. C3d or C3dg was added in increasing amounts. At the same time, a positive control wherein staphylococcal extracellular fibrinogen-binding protein (Efb), a known C3d ligand from the bacterium *Staphylococcus aureus*, was also immobilized and added with increasing amounts of C3d or C3dg. Bound C3d or C3dg was detected by rabbit polyclonal C3d antiserum. C3d bound to Tef1p and the binding was dose-dependent (**Figure 13C**). However, more C3d bound to staphylococcal Efb than to Tef1p. Approximately 44% more C3d bound to Efb than to Tef1p at a C3d concentration of 5.0 µg/ml. C3d did not bind to immobilized BSA, which was used as negative control. The C3b degradation product C3dg also bound to immobilized Tef1p and the binding was dose-dependent (**Figure 13D**). Twenty-two percent more C3dg bound to Efb than to Tef1p at 0.5 µg/ml C3dg concentration.

To further confirm the specificity of the Tef1p-C3d interaction, the amount of Factor H which bound to Tef1p-complexed C3d was measured in ELISA. At first, C3d, in increasing amounts, was added to immobilized Tef1p. Then, Factor H was added in constant amount to Tef1p-complexed C3d after extensive washing. Bound Factor H was detected by goat polyclonal anti-Factor H antibody. Factor H bound to Tef1p-complexed C3d and the binding was dose-dependent. This confirms that C3d specifically binds to *Candida* Tef1p and can mediate the generation of a tripartite complex between Tef1p, C3d and Factor H (**Figure 13E**). Taken together, this demonstrates that *Candida* Tef1p binds the complement regulators Factor H and C4BP, and the C3b degradation products C3d and C3dg.

3.2.3. *Candida* Tef1p binds plasminogen

Tef1p was identified to be one of the surface proteins of *C. albicans* which bind the plasma protein plasminogen [9,37]. To confirm the binding of plasminogen to *Candida* Tef1p, Tef1p was immobilized and plasminogen was added in increasing amounts. Bound plasminogen was detected using goat polyclonal anti-plasminogen in ELISA. Plasminogen bound to Tef1p and the binding was dose-dependent (**Figure 14A**). The Tef1p-plasminogen interaction was further confirmed with the use of surface plasmon resonance (SPR). Plasminogen was applied as analyte and association to immobilized Tef1p was measured. Plasminogen, at concentrations of 100-400 nM, bound to Tef1p in a dose dependent manner (**Figure 14B**). The Tef1p-plasminogen complex was stable and had a slow dissociation profile.

Plasminogen interaction with other *C. albicans* surface proteins such as Gpm1p and Pra1 are known to involve lysine residues [61,62,116]. To determine if this is also the case for Tef1p, the lysine analog ε-aminocaproic acid (ε-ACA) was used in an inhibition assay using ELISA. Plasminogen was incubated with ε-ACA in increasing amounts and added to immobilized Tef1p. Bound plasminogen was detected using the goat polyclonal anti-plasminogen

antibody. ϵ -ACA inhibited plasminogen binding to Tef1p and the inhibition was dose-dependent (**Figure 14C**). ϵ -ACA inhibited 58% of the binding at 0.4 mM concentration. However, the same inhibition level was reached even with the use of 1.0 mM ϵ -ACA. The ϵ -ACA-mediated inhibition of Tef1p-plasminogen interaction was confirmed in SPR analysis. Plasminogen, preincubated with ϵ -ACA in increasing amounts, was used as analyte and association with immobilized Tef1p was measured. As expected, ϵ -ACA inhibited plasminogen binding to Tef1p and the inhibition was dose-dependent. ϵ -ACA, at a concentration of 2.0 nM could totally abolish the interaction between plasminogen and Tef1p (**Figure 14D**). Thus, lysine residues are involved in the interaction between Tef1p and plasminogen.

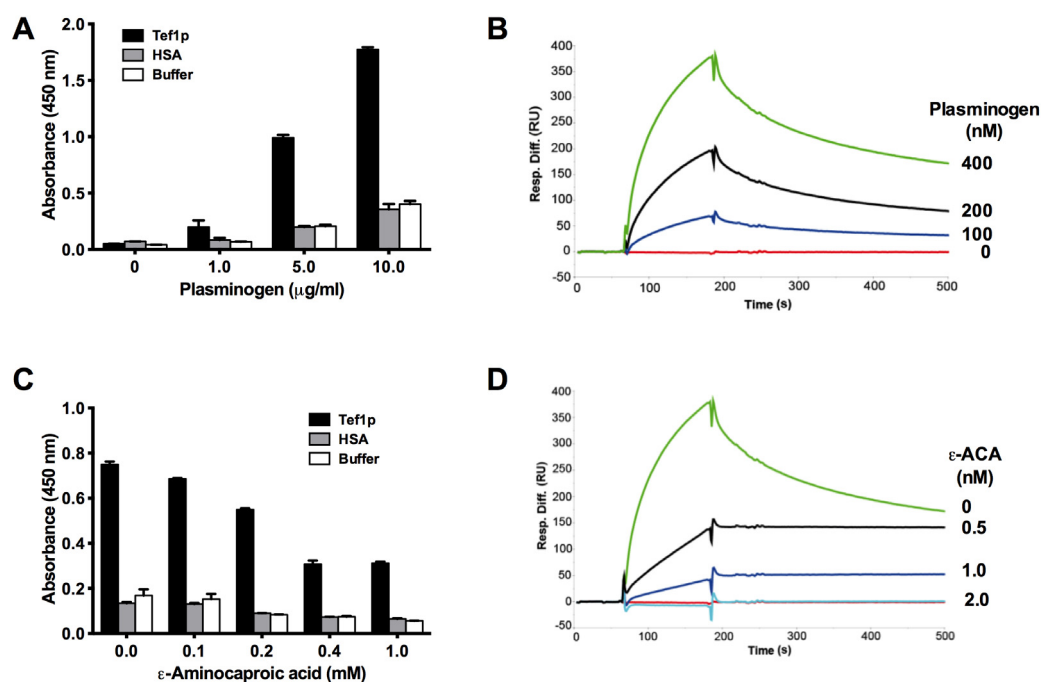


FIGURE 14. Plasminogen binds to Tef1p

Plasminogen binds to Tef1p in a dose-dependent manner. Binding of plasminogen to Tef1p was assayed in ELISA (A). Tef1p was immobilized on microtiter plate and plasminogen was added at indicated concentrations. Bound plasminogen was detected by goat plasminogen antiserum and HRP-conjugated rabbit anti-goat IgG. The dose-dependent binding of plasminogen to Tef1p was confirmed by surface plasmon resonance (SPR) analysis (B). Tef1p was immobilized on the chip surface, and plasminogen was used as the analyte in fluid phase at indicated concentrations. Lysine residues are involved in the interaction between Tef1p and plasminogen. The effect of the lysine analog ϵ -ACA on plasminogen binding to Tef1p was assayed in ELISA (C). Plasminogen with ϵ -ACA (in indicated concentrations) was added to immobilized Tef1p on microtiter plate. Bound plasminogen was detected as in A. ϵ -ACA reduced the binding of plasminogen to Tef1p in a dose-dependent manner. The effect of ϵ -ACA on plasminogen binding to Tef1p was confirmed in SPR analysis (D). Tef1p was immobilized on the chip surface, and plasminogen with ϵ -ACA (in indicated concentrations) were applied in the fluid phase as analyte. HSA was used as negative protein control for A and C. Buffer was used as antibody control. Data of A and C are mean \pm SD (error bars) of three independent experiments. Data of B and D are representatives of 3 independent experiments.

3.2.4. Tef1p-bound plasminogen is converted to functionally active plasmin

Plasmin is a protease that degrades fibrinogen, ECM proteins and the complement component C3b [9]. To determine if an active plasmin is generated from Tef1p-bound plasminogen, degradation of different substrates was determined in a series of plasminogen activation assays. To this end, plasminogen was bound to immobilized Tef1p, and after extensive washing, the tissue-type plasminogen activator (tPA) was added together with the substrate (chromogenic S-2251, fibrinogen, vitronectin, or the complement component C3b). Following incubation, degradation of substrate was monitored in a time-dependent manner. Tef1p-bound plasminogen could be activated to plasmin and degrade the synthetic substrate S-2251. An exponential change in absorbance, which is proportional to plasmin activity that produces chromogenic degradation product, was observed for almost 15 h before the degradation profile started to remain constant at Absorbance_{450 nm} = 1.2 (**Figure 15A**). The fluid-phase plasminogen could be activated to plasmin in a faster rate where the S-2251 substrate was readily degraded in the first 4 h before reaching a constant Absorbance_{450 nm} = 1.0. The negative control HSA did not bind plasminogen, so no change in absorbance was observed. Also, no change in absorbance was measured in samples lacking plasminogen or tPA in the reaction mixture.

The degradation activity of Tef1p-bound plasminogen was also assayed using its natural substrates fibrinogen and vitronectin. To this end, plasminogen was bound to immobilized Tef1p and after extensive washing, tPA and the substrate fibrinogen or vitronectin were added. After incubation, the proteins in the reaction mixture were separated in SDS-PAGE, transferred onto a membrane, and fibrinogen or vitronectin was detected by Western blotting. Tef1p-bound plasminogen, when activated to plasmin, degraded fibrinogen (**Figure 15B**). Cleavage of both 55- and 72-kDa fibrinogen bands was seen after two hours. The positive control plasminogen when activated with tPa in fluid phase completely degraded fibrinogen, as evidenced by the disappearance of the fibrinogen bands (**Figure 15B, Lane 4**).

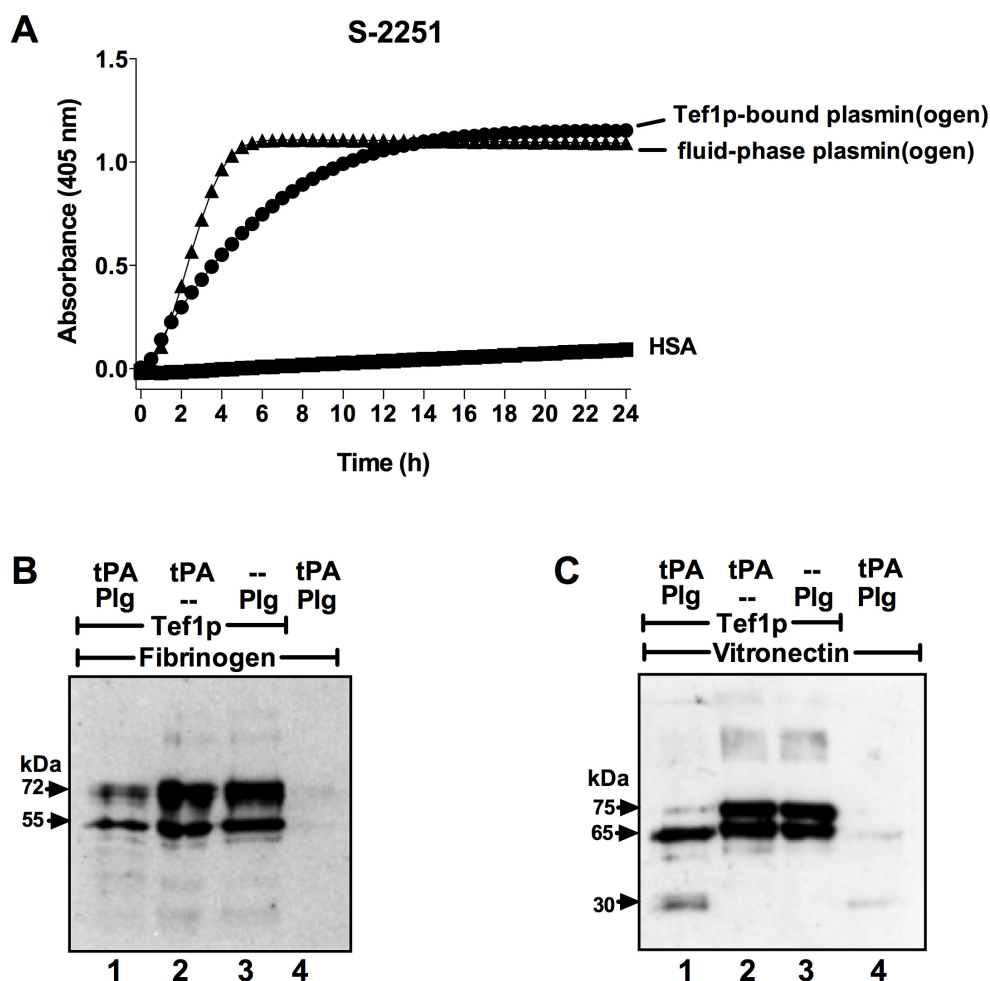


FIGURE 15. Plasminogen bound to Tef1p is functionally active

(A) Plasminogen was bound to Tef1p that was immobilized on microtiter plate. After extensive washing, the activator tPA (4 ng/well) together with chromogenic substrate S-2251 were added, and the rate of product formation was monitored for 24 h by absorbance at 405 nm. The rate of product formation is proportional to the activity of plasmin(ogen). Assay performed with fluid-phase plasminogen was used as positive control while plasminogen added to immobilized HSA was used as negative control. Degradation activity of Tef1p-bound plasmin(ogen) was done in an assay using the natural substrates fibrinogen (B) and vitronectin (C). Plasminogen (was bound to immobilized Tef1p and after washing, tPA together with the substrate were added (B and C, Lane 1). Samples were collected after two hours, loaded on 10% SDS-PAGE gel, transferred onto a nitrocellulose membrane, and degradation was determined by Western blotting using rabbit fibrinogen (B) or vitronectin (C) antiserum. Plasminogen in fluid phase added with tPA was used as positive control (B and C, Lane 4). Assays performed in the absence of plasminogen (B and C, Lane 2) or tPA (B and C, Lane 3) were used as negative controls. Molecular weights of degradation products are indicated.

Tef1p-bound plasminogen, when activated to plasmin, also degraded vitronectin (**Figure 15C**). Vitronectin degradation was identified by the disappearance of the 75-kDa band and the appearance of approximately 30-kDa degradation product. The positive control plasminogen when activated with tPa in fluid phase completely degraded vitronectin as evidenced by the disappearance of both the 65- and 75-kDa vitronectin bands (**Figure 15C**, Lane 4). The lack of tPa (**Figures 15B and 15C, Lane 2**) or plasminogen (**Figures 15B and 15C, Lane 3**) did not degrade the substrates as demonstrated by the intact protein bands. Overall, plasminogen when bound to Tef1p, can be activated to the protease plasmin and maintain its degradation activity.

3.2.5. Tef1p-bound plasminogen cleaves the complement component C3b

It was recently shown that plasminogen degrades the complement component C3b [9]. To determine if the Tef1p-bound plasminogen degrades C3b, first, plasminogen was bound to immobilized Tef1p and after washing, tPA and C3b were added. Next, samples were collected at specific intervals for 4 hours. C3b cleavage was determined in Western blot by the appearance of the cleavage products α' 43- and α' 41-kDa bands. The Tef1p-bound plasminogen cleaved C3b in a time-dependent manner with the appearance of the early cleavage products after two hours (**Figure 16A, Lane 2**). C3b cleavage products increased after 3 and 4 hours (**Figure 16B, Lanes 3 and 4**) as seen in the significant increase in the intensities of the cleavage product bands. The appearance of C3b cleavage products in the blot where the assay was performed in fluid phase was used as reference (**Figure 16B**). No C3b cleavage products were seen in samples without plasminogen, tPA or Tef1p (**Figure 16C; Lanes 1, 2 and 3, respectively**). Taken together, Tef1p-bound plasminogen is activated to plasmin and cleaves C3b.

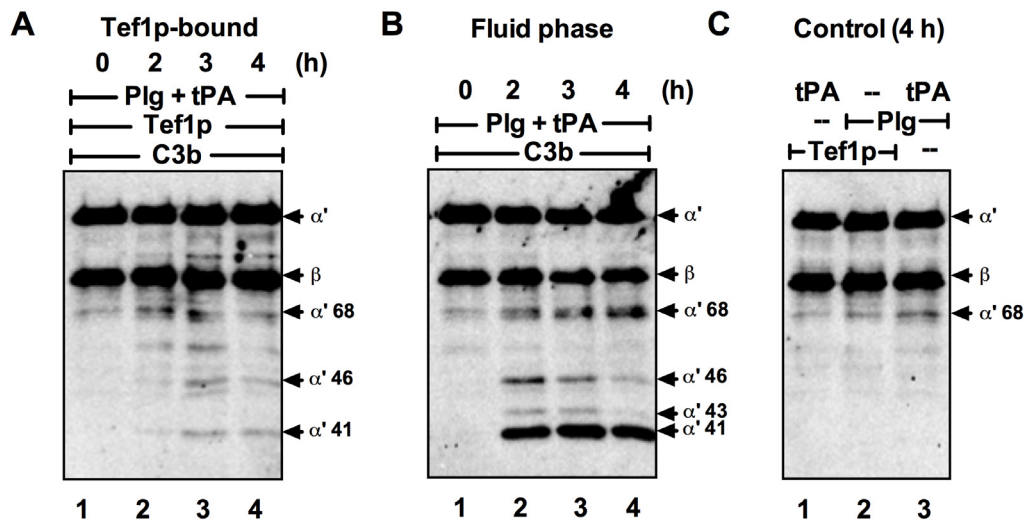


FIGURE 16. Tef1p-bound plasminogen cleaves the complement component C3b

Plasminogen was added to immobilized Tef1p on microtiter plate. After extensive washing, the activator tPA (4 ng/well) and the substrate C3b (5 μ g/ml) were added. Samples from the reaction mixture were collected at indicated times, separated by SDS-PAGE, transferred onto a nitrocellulose membrane, and C3b cleavage products were identified in Western blot using goat C3 antiserum and HRP-conjugated rabbit anti-goat IgG. C3b is identified by the α' -chain and the β -chain. C3b degradation is visualized by the appearance of the cleavage products as $\alpha'68$, $\alpha'43$, and $\alpha'41$ fragments (A and B, Lanes 2-4). Degradation of C3b by activated fluid phase plasminogen was used as positive control (B). Reaction mixtures without plasminogen (C, Lane 1), tPA (C, Lane 2) or Tef1p (C, Lane 3) were used as negative controls.

3.2.5. Tef1p is expressed on the surface of *C. albicans*

For *Candida* Tef1p to bind Factor H and plasminogen, it must be secreted and/or present on the fungal cell surface. To determine the Tef1p expression on the surface of *C. albicans*, Tef1p-specific antiserum was first generated against Tef1p by immunizing rabbits with the purified recombinant Tef1p. The generated rabbit Tef1p antiserum detected the recombinant protein in Western blot as 55-kDa band in Western blot (**Figure 17A, Lane 1**). The same band was detected by the mouse monoclonal antibody reacting with penta-His (**Figure 17A, Lane 3**). The Tef1p band was not detected with pre-immune serum (**Figure 17A, Lane 2**).

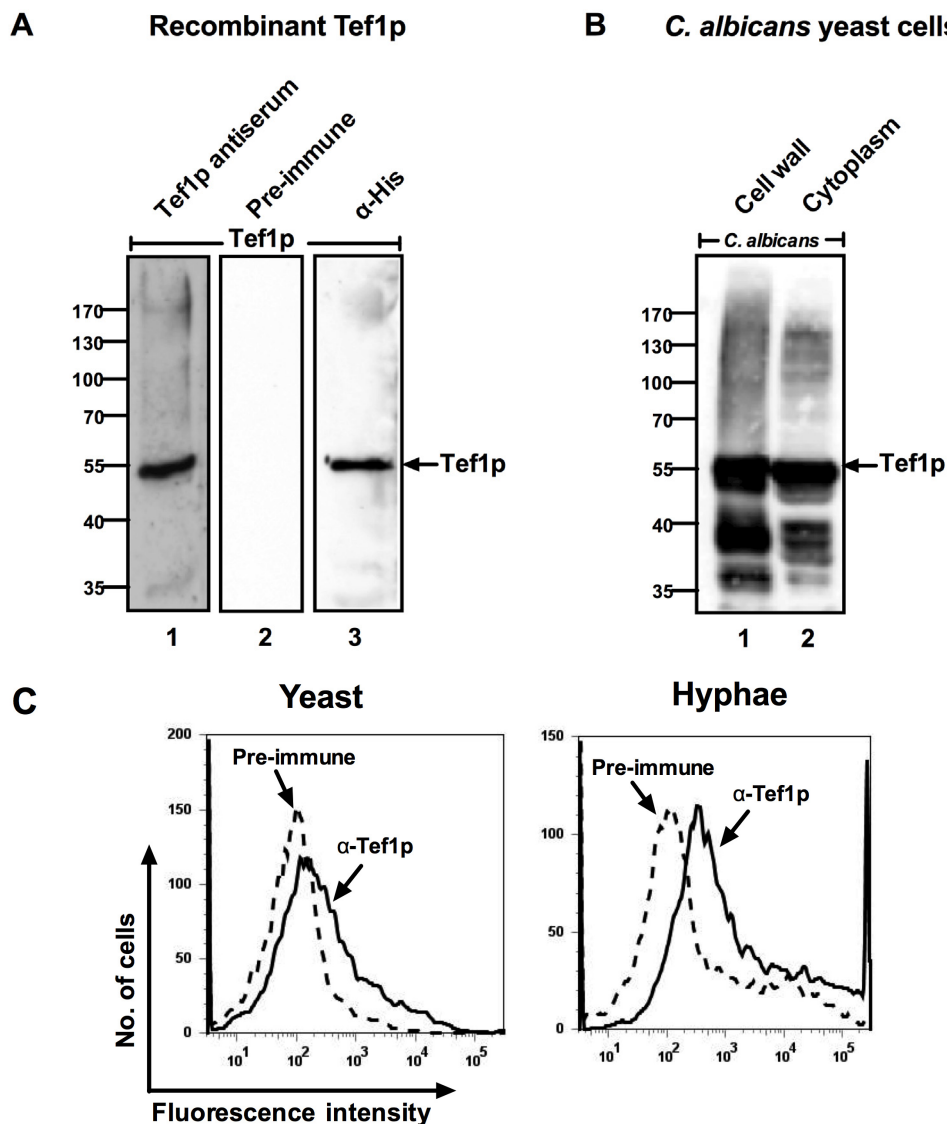


FIGURE 17. Tef1p is expressed on the surface of *C. albicans*

Tef1p antiserum (α -Tef1p) was generated in rabbit using recombinant Tef1p. (A) Recombinant Tef1p was loaded on SDS-PAGE gel and transferred onto nitrocellulose membrane for detection in Western blotting using rabbit pre-immune serum (Lane 2), rabbit Tef1p antiserum (Lane 1) or monoclonal mouse penta-His IgG (α -penta-His) (Lane 3). Rabbit pre-immune serum or Tef1p antiserum was detected by HRP-conjugated goat anti-rabbit IgG. α -penta-His was detected by HRP-conjugated goat anti-mouse IgG. (B) *C. albicans* cell wall (Lane 1) and cytoplasm (Lane 2) fractions were loaded on SDS-PAGE, transferred onto nitrocellulose membrane and analyzed by Western blotting for the presence of native Tef1p using α -Tef1p and HRP-conjugated goat anti-rabbit IgG. The migration of the molecular weight protein standards and the Tef1p band are indicated. (C) Tef1p is present on yeast and hyphal cells of *C. albicans*. Yeast or hyphal cells were incubated with α -Tef1p or pre-immune serum, followed by Alexa Fluor® 647-conjugated goat anti-rabbit IgG, and fluorescence was measured by flow cytometry.

D

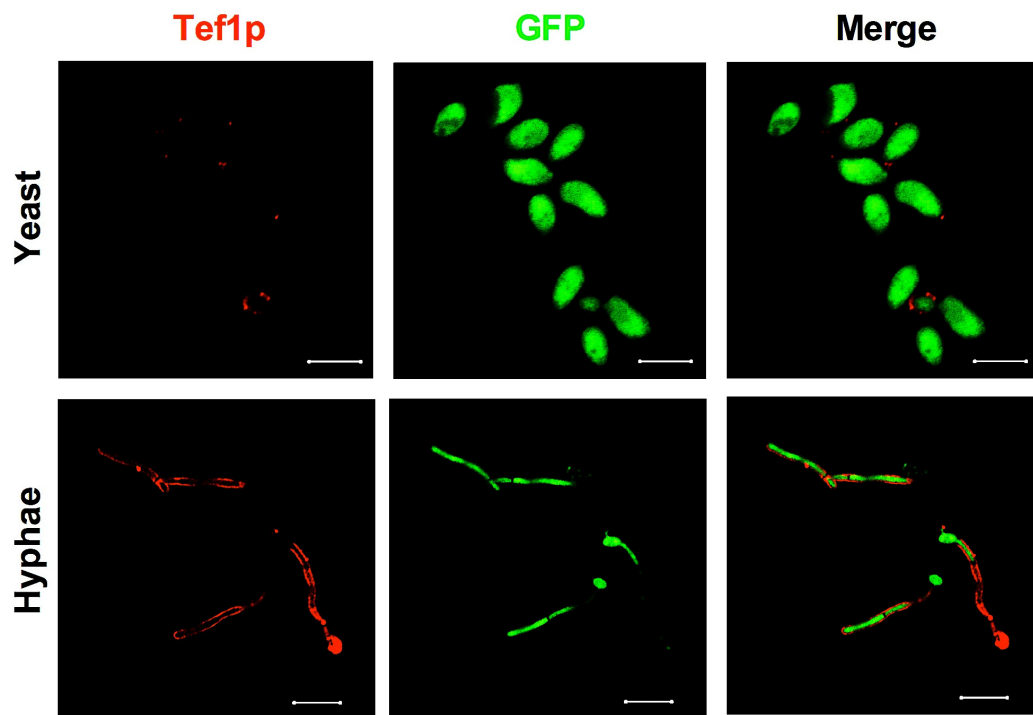


FIGURE 17. Tef1p is expressed on the surface of *C. albicans* (continued)

(D) Expression of Tef1p on surface of *C. albicans* was visually confirmed in LSM. GFP-expressing yeast (top panel) or hyphal (bottom panel) cells (green) were attached onto a coverslip and fixed with 3% paraformaldehyde. After washing, cells were incubated with α -Tef1p and Alexa Fluor® 647-conjugated goat anti-rabbit IgG (red). Scale bar = 10 μ m (top panel) or 20 μ m (bottom panel).

The Tef1p native form was detected in samples from cell wall and cytoplasm of *C. albicans* SC5314 using the generated Tef1p antiserum in Western blot. Tef1p was detected in both cell wall and cytoplasm as a 55-kDa band along with several other bands (**Figure 17B**). The other bands with less than 55-kDa molecular mass could be Tef1p degradation products while the bands with more than 55-kDa molecular mass could be Tef1p complexed with other cellular proteins (**Figure 17B, Lanes 1 and 2**).

Aside from confirming the cellular localization of Tef1p, we also compared the expression of the protein on the surface of yeast and hyphae forms of *C. albicans*. The surface expression of Tef1p in both cellular forms was measured by flow cytometry and visually confirmed by laser scanning microscopy using the rabbit Tef1p antiserum. Yeast and hyphal cells expressed Tef1p on their surfaces. However, more Tef1p was detected on the surface of hyphal cells (MFI=513) than yeast cells (MFI=16) (**Figure 17C**). Visually, Tef1p expression on yeast cells was seen only at the budding part of the cells (**Figure 17D, top panel**), whereas the Tef1p expression on hyphal

cells was observed all throughout the surface of the hyphae (**Figure 17B, bottom panel**) indicating that Tef1p is mainly expressed on hyphal form of the fungus.

3.2.6. Generation of *C. albicans* *TEF1* knock-out and revertant strains

To determine the role of Tef1p on the binding of *C. albicans* to proteins of the innate immune system, *tef1Δ/Δ* deletion mutant strain was generated using the PCR-based gene disruption technique [116,117]. Strains BWP17 (wild-type), *tef1Δ* heterozygous deletion mutant, and *tef1Δ/Δ* homozygous deletion mutant were genetically analyzed for the correct deletion of *TEF1* by PCR and Southern blot analyses. For the PCR analysis, two sets of primers were used to specifically amplify *TEF1*. The first primer pair amplified the region between the upstream flanking region (*TEF1-disrptnup*) and middle of the *TEF1* gene sequence (*TEF1_2*) while the other pair amplified the region from the middle of the *TEF1* gene sequence (*TEF1_3*) and downstream flanking region (*TEF1-disrptndown*). The expected PCR product sizes of 927 bp and 924 bp were seen in WT and *tef1Δ* but not in *tef1Δ/Δ* (**Figure 18A**), indicating the deletion of the *TEF1* gene in the *tef1Δ/Δ* deletion mutant.

Confirmation of *TEF1* deletion was done using Southern blotting, wherein 517 base-pair (bp) PCR product amplified by the primers CaTEF1Int-Fwd and CaTEF1Int-Rev from *C. albicans* SC5314 genomic DNA, was used as a probe on *Pci*I-digested genomic DNA (**Figure 18B**). The expected size of 6282 bp was detected in WT and *tef1Δ*, with the latter having only a faint band since it only had one copy of the *TEF1* gene (**Figure 18C**). No band was seen in *tef1Δ/Δ* at the expected size. However, since the *TEF2* gene shares 99% identity with *TEF1*, another band was detected at the size of 2218 bp in all samples. Overall, the *TEF1* gene was successfully deleted in *C. albicans*.

To obtain the *tef1Δ/Δ::TEF1*-reintegration strain, Clp10-*TEF1* plasmid was used to transform the uridine auxotrophic *C. albicans* strain *tef1Δ/Δura⁻*. Integration of the plasmid was confirmed in PCR by amplifying the region between *URA3* and the flanking region of RP10 locus using the primers *URA3-F2* and *RPF-F1*. A PCR product was seen in the *TEF1* reintegration strain while no band was seen in the *tef1Δ/Δ* deletion mutant, indicating the successful reintegration of *TEF1* (**Figure 18D**).

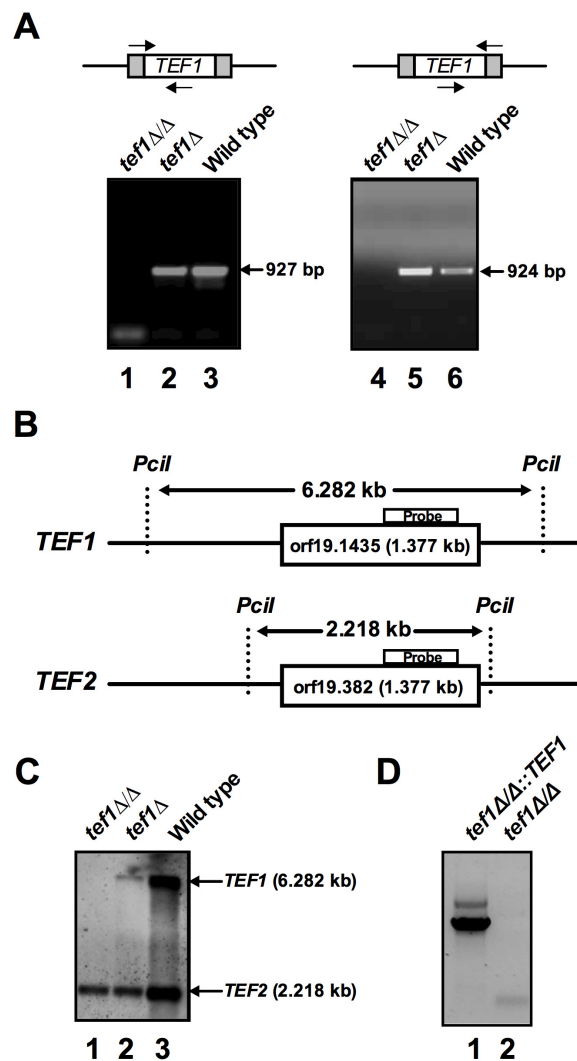


FIGURE 18. Generation of *C. albicans* *TEF1* knock-out and revertant strains.

(A) Successful deletion of both gene copies of *TEF1* was determined by PCR using primers which amplify the region between upstream flanking region and middle of ORF or middle of ORF and downstream flanking region of *TEF1* (orf19.1435). The gene sequences were amplified in wild type (Lanes 3 and 6) and *tef1* Δ heterozygous deletion mutant (Lanes 2 and 5) but not in *tef1* Δ/Δ homozygous deletion mutant (Lanes 1 and 4). The sizes of the amplified gene products are indicated. (B) Schematic representation of the Southern blotting strategy used to confirm the deletion of *TEF1* in *tef1* Δ/Δ . A 517-bp probe was amplified from genomic DNA of *C. albicans* SC5314. The probe was used on *Pcil*-digested genomic DNA. The expected band sizes are indicated for *TEF1* and *TEF2*, which shares 99% identity with *TEF1*. (C) Southern blot confirming the deletion of *TEF1*. Strains *tef1* Δ/Δ (Lane 1), *tef1* Δ (Lane 2) and SC5314 (wild type, Lane 3) were analyzed. Sizes of detected *TEF1* and *TEF2* are indicated. (D) Confirmation of the reintegration of *TEF1* using PCR. The correct reintegration of *TEF1* in RP10 locus of the genomic DNA of *tef1* $\Delta/\Delta::TEF1$ reintegration strain and *tef1* Δ/Δ deletion mutant was determined by amplifying the region between *URA3* and RP10 locus flanking region.

3.2.7. Characterization of *C. albicans* *TEF1* knock-out mutant

Translation elongation factor 1-alpha is possibly an essential gene in *C. albicans* [87]. To determine if deletion of *TEF1* affects the viability of *C. albicans*, the *tef1Δ/Δ* deletion mutant was grown in liquid or on solid YPD medium and the growth was monitored at different incubation conditions. The *tef1Δ/Δ* deletion mutant grew in YPD broth after 14 h at 30 °C at the same rate as the wild type and the *tef1Δ/Δ::TEF1*-reintegration strain (**Figure 19A**). In addition, *tef1Δ/Δ* deletion mutant grew on YPD agar at 30 °C and 37 °C after 72 h of incubation in the same manner as the wild type and revertant strain (**Figure 19B**). Taken together, deletion of *TEF1* in *C. albicans* does not affect the growth of the fungus, indicating that *TEF1* is a non-essential gene. Since Tef1p is expressed on the surface of *C. albicans*, the effect of various cell wall disturbing agents and stresses on the growth of *tef1Δ/Δ* deletion mutant was investigated. The cell wall perturbing agents calcofluor white (7 nM), congo red (20 µg/ml), and SDS (0.004%) did not affect growth of *tef1Δ/Δ* deletion mutant in YPD at 30 °C for 72 h (**Figure 19C**). In the same manner, the *tef1Δ/Δ* mutant had the same growth as the wild type and *tef1Δ/Δ::TEF1*-reintegration strain in NaCl (1.5 M) and D-sorbitol (1.0 M), indicating that *TEF1* is not necessary in osmotic stress resistance of *C. albicans* (**Figure 19D**). The oxidative stressors menadione (25 µM), H₂O₂ (2.0 mM), and t-butyl hydroperoxide (t-BOOH, 2.0 mM) had no effect on the growth of *tef1Δ/Δ* deletion mutant (**Figure 19E**). In conclusion, deletion of *TEF1* does not affect the cell wall integrity of *C. albicans* and its adaptation to osmotic and oxidative stress. However, deletion of *TEF1* made *C. albicans* sensitive to the antifungal hygromycin B (0.5 mg/ml) and reintegration of *TEF1* in *tef1Δ/Δ* deletion mutant restored the growth (**Figure 19F**). Thus, *TEF1* contributes to the resistance of *C. albicans* against an antibiotic whose mode of action is to inhibit protein synthesis.

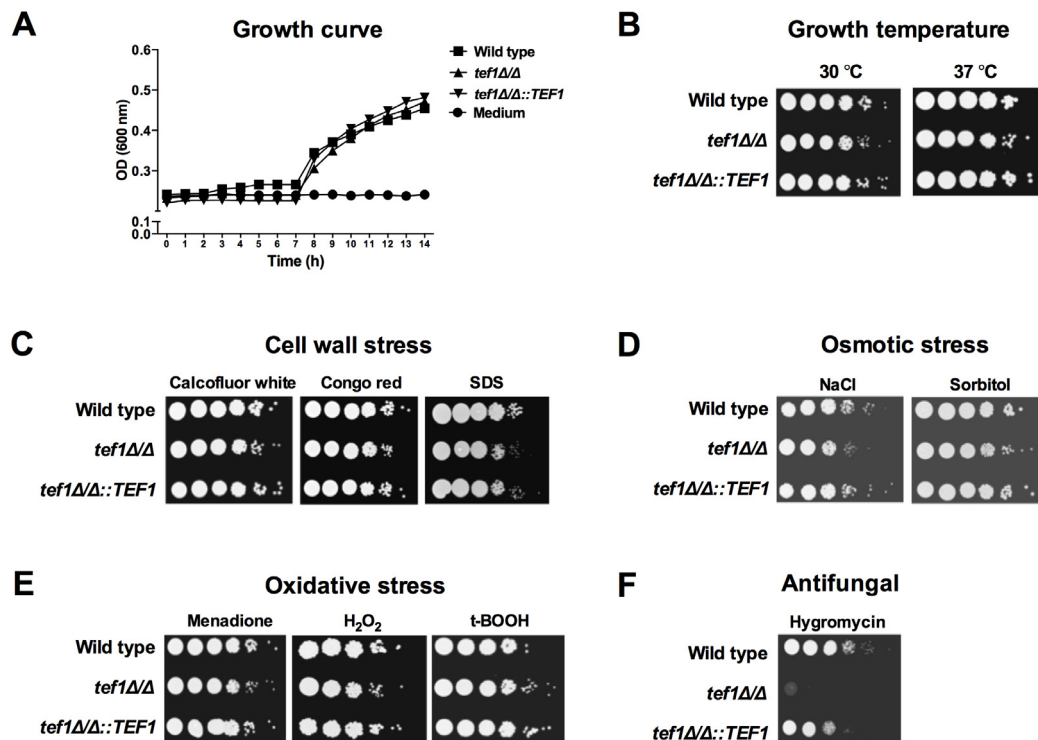


FIGURE 19. Characterization of *TEF1* homozygous deletion mutant

Growth rate of *C. albicans* SC5314 (wild type), *tef1Δ/Δ* deletion mutant, and *tef1Δ/Δ::TEF1* reintegration mutant in YPD broth at 30 °C was monitored for 14 h by collecting samples every hour and measuring the optical density (OD) at 600 nm. (B) Ten-fold serial dilutions of *C. albicans* SC5314 (wild type), *tef1Δ/Δ*, and *tef1Δ/Δ::TEF1* were spotted on YPD agar and incubated for 2-3 days at 30 °C or 37 °C. Growth of strains under different environmental stresses was determined in spot test analysis. Ten-fold serial dilutions of wild type, *tef1Δ/Δ*, and *tef1Δ/Δ::TEF1* were spotted on YPD agar with different stress conditions including (C) cell wall stress [calcofluor white (6.7 nM), congo red (20 μg/ml), and SDS (0.004%)], (D) osmotic stress [NaCl (1.5 M) and sorbitol (1.0 M)], and (E) oxidative stress [menadione (25 μM), H₂O₂ (2 μM), and t-BOOH (2 mM)]; and (F) antifungal hygromycin (0.5 mg/ml). Plates were incubated for 2-3 days at 30 °C. Experiments were repeated at least twice yielding similar results. Representative pictures are shown.

3.2.8. Deletion of *TEF1* decreases binding of plasminogen and C3d on *C. albicans*

Because Tef1p binds components of the complement system, the effect of the deletion of *TEF1* on the binding of Factor H, C4BP, C3d and plasminogen by *C. albicans* was analyzed. To this end, purified Factor H, C4BP, C3b or plasminogen was bound to the *tef1Δ/Δ* deletion mutant, and bound proteins were detected with appropriate antisera in flow cytometry. Factor H bound *tef1Δ/Δ* deletion mutant in the same manner as the wild type and *tef1Δ/Δ::TEF1*-reintegration strain (data not shown). In addition, the same amount of C4BP bound to *tef1Δ/Δ* deletion mutant compared to the wild type and *tef1Δ/Δ::TEF1*-reintegration strain (data not shown).

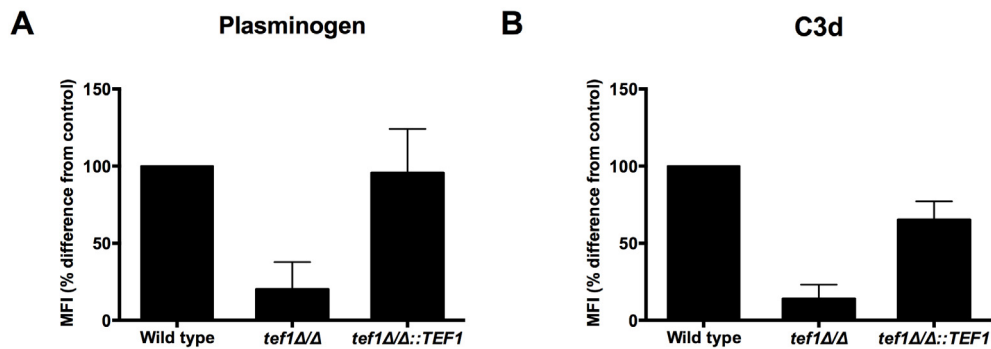


FIGURE 20. Deletion of *TEF1* decreases binding of plasminogen and C3d to *C. albicans*
Binding of plasminogen (A) or C3d (B) to wild type, *tef1Δ/Δ* deletion mutant, and *tef1Δ/Δ::TEF1* reintegration strain was determined by flow cytometry. Yeast cells were incubated with plasminogen or C3d. Bound plasminogen was detected by goat plasminogen antiserum followed by Alexa Fluor® 647-conjugated rabbit anti-goat IgG. Bound C3d was detected by rabbit C3d antiserum followed by Alexa Fluor® 647-conjugated goat anti-rabbit IgG. Data are reported as % difference from antibody control. Bars are representatives of 3 independent experiments.

However, plasminogen and C3d bound less to the *tef1* knock-out strain. The *tef1Δ/Δ* deletion mutant bound 67.5% less plasminogen in comparison to the wild type, and 71.9% less in comparison to the *tef1Δ/Δ::TEF1* reintegration strain (**Figure 20A**). In addition, *tef1Δ/Δ* deletion mutant bound 92.6% and 87% less C3d than the wild type and *tef1Δ/Δ::TEF1* reintegration strain, respectively (**Figure 20B**). Taken together, deletion of *TEF1* decreases the ability of *C. albicans* to bind the plasma protein plasminogen and complement component C3d but not Factor H and C4BP.

3.2.9. *C. albicans* Tef1p interacts with human neutrophils

Neutrophils, as central components of the innate immune system, directly attack *C. albicans* [28]. To determine the role of Tef1p on the interaction of *C. albicans* with human neutrophils, Tef1p was added to neutrophils and bound Tef1p was measured using flow cytometry. Tef1p bound to freshly isolated human neutrophils with an MFI of 467.8 compared to the antibody control with an MFI of 167.1 (**Figure 21A**). Thus, *Candida* Tef1p binds to human neutrophils.

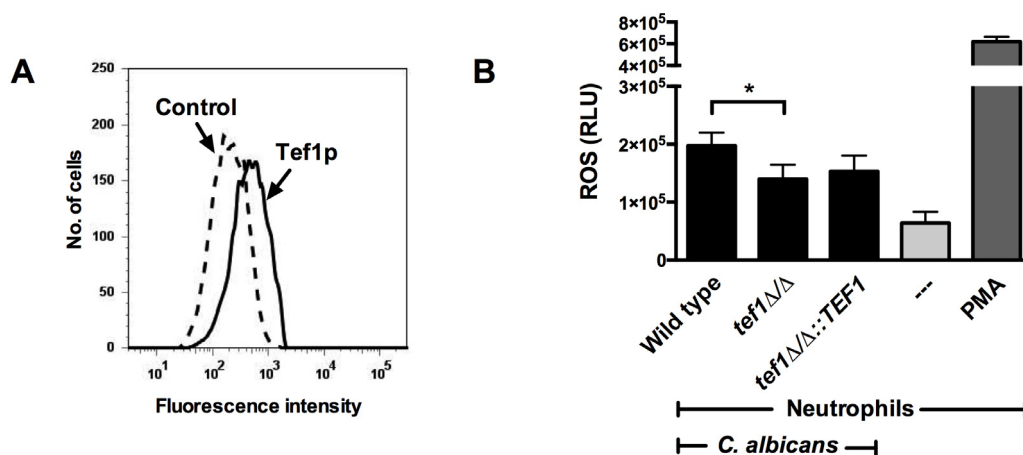


FIGURE 21. *C. albicans* Tef1p interacts with neutrophils

Tef1p binds to neutrophils. Tef1p (10 μ g) was incubated with freshly-isolated PMNs from human peripheral blood. Bound Tef1p was quantified using rabbit Tef1p antiserum and Alexa Fluor® 647-conjugated goat anti-rabbit IgG in flow cytometry. Cells were incubated with Fc receptor blocker prior to addition of primary antibody. Cells without Tef1p were used as control. (B) Deletion of *TEF1* in *C. albicans* reduces the reactive oxygen species (ROS) response by PMNs. ROS release by PMNs incubated with *C. albicans* strains was measured using luminol-based chemiluminescence. *C. albicans* SC5314 (wild type), *tef1Δ/Δ* deletion mutant, or *tef1Δ/Δ::TEF1* reintegration strain was added to neutrophils at MOI=1 and incubated at 37 °C with 5% CO₂, recording the chemiluminescence every 2.5 min for 2.5 h. PMNs incubated with or without phorbol 12-myristate 13-acetate (PMA) was used as positive or negative control, respectively. Data are reported as mean of areas under the curve in relative luminiscence units (RLU). Error bars represent SD of three independent experiments. *P<0.01 compared with the wild type strain.

Release of reactive oxygen species (ROS) by human neutrophils is an effector mechanism to directly kill invading microorganisms [34,117]. Because Tef1p binds to neutrophils, and as fungal surface proteins contribute to the interaction between *C. albicans* and neutrophils, the amount of released ROS induced by *C. albicans* *tef1* knock-out strain was measured with the use of luminol-based chemiluminescence. A significantly reduced amount of ROS released by the human neutrophils was induced by the *C. albicans* *tef1* knock out strain. When *tef1Δ/Δ* deletion mutant was cocultivated with neutrophils, the neutrophils released 29% less ROS in comparison to the wild type and 8.7% less than that of *tef1Δ/Δ::TEF1*-reintegration strain (**Figure 21B**). This suggests that Tef1p contributes the recognition of *C. albicans* by neutrophils or induces a specific receptor on the surface of neutrophils which activates the release of ROS.

4. DISCUSSION

Host-pathogen interactions are governed by several mechanisms in which both players try to maintain a balance to co-exist. On the one hand, microbial pathogens have evolved a plethora of strategies to survive in its chosen niche inside the human body. On the other hand, the human host has developed itself with various means to counteract the undesirable effects of the foreign microbial pathogens. However, when one of the players gets compromised, the other dominates and causes destructive consequences. Such is the case when the human body acquires a defect in its machineries to control growth of fungal pathogens, like neutropenia. In this situation, fungal pathogens such as *Candida albicans* disseminate in the human body and cause damage to human tissues which could lead to dysfunction of the whole human body.

Candida albicans is a leading fungal pathogen which causes death in humans. *C. albicans* possesses several virulence factors and has developed numerous mechanisms to evade the recognition and attack of the human body. Virulence factors can be secreted or surface-bound which *C. albicans* uses to start the infection process. During infection, the fungal pathogen uses several strategies to evade the attack of the human immune system either by making itself unrecognizable or degrading components of the human effector mechanisms.

The current prevalence of *C. albicans* infections in humans and the high mortality rate caused by the fungal pathogen indicate that the pathogenesis is still not well understood. It was therefore the aim of this study to contribute to the understanding of the *C. albicans*-human host interplay with the hope that novel and effective ways could be discovered to inhibit the detrimental effects of *C. albicans* infection and improve quality of life. To achieve this, the work presented here investigated the roles of two surface proteins of *C. albicans* in the human tissue invasion and immune evasion.

4.1. *C. albicans* phosphoglycerate mutase (Gpm1p) as a surface protein which mediates adhesion to human endothelial cells and keratinocytes using vitronectin as ligand

Attachment of *Candida albicans* to human tissues greatly contributes to the virulence of the fungal pathogen in starting the infection process. Thus, it is important to know the mechanism by which *C. albicans* attaches to human cells prior to tissue invasion. It was therefore the aim of this work to describe the role of a specific surface protein on the direct contact of *C. albicans* with human cells, and identify the receptor on the surface of human cells which is responsible for the interaction.

In this study, the glycolytic enzyme Gpm1p, is identified to mediate the adhesion of the fungal pathogen *C. albicans* to the human cells. It was initially investigated if Gpm1p binds to human cells. Here, Gpm1p binds to endothelial cells and keratinocytes, but not to monocytes. This has a significant implication on the role of Gpm1p as a surface protein of *C. albicans*. It specifically identifies the protein as an adhesin for tissue adherence and not as a ligand for phagocytes which might lead to recognition and phagocytosis of the fungus. The role of Gpm1p in the binding of *C. albicans* to endothelial cells was confirmed by the decrease in the association (adherence and/endocytosis) of the *GPM1* homozygous deletion strain to HUVEC.

The lack of *GPM1* decreases the ability of *C. albicans* to associate with human cells, but not totally abolishes it. This can be attributed to the fact that other surface proteins of *C. albicans* exist which can mediate the binding to endothelial cells such as Als3 [118,119] and Ssa1 [34,120,121]. To eliminate the effects of other surface proteins present on *C. albicans*, Gpm1p was coated on latex beads and association with endothelial cells was analyzed. Gpm1p mediated the association of the latex beads with the human endothelial cells. Thus, Gpm1p alone causes adhesion or/and endocytosis to and by endothelial cells which makes Gpm1p a relevant adhesin and/or invasin for endothelial cells.

Candida Gpm1p binds to human keratinocytes. This is relevant since *C. albicans* is one of the leading pathogens to cause cutaneous infections. Gpm1p is identified as surface protein that adheres to skin cells which could lead to further invasion of the epidermal layers during skin infection. It is established that *C. albicans* binds to keratinocytes [68,119]. However, at present, no surface protein has been identified which directly mediates contact between *C. albicans* and keratinocytes. Previous works have only identified mannose and phospholipomannan on the cell wall of *C. albicans* which triggers immune response by keratinocytes [120,121]. Thus, I identify Gpm1p as a new *C. albicans* surface protein which directly interacts with human keratinocytes.

Screening for the possible ligand present on human cells led to the identification of the adhesive glycoprotein vitronectin. This work specifically identifies Gpm1p to be a vitronectin-binding protein of *C. albicans*. It is further demonstrated in this study, through binding and blocking assays, that heparin-binding sites (HBS) on the vitronectin molecule are involved in the interaction between Gpm1p and vitronectin. This is in line with the results of Limper et al (1994) which showed that *C. albicans* cells bind to the glycosaminoglycan-binding region of vitronectin [68]. Interestingly, the same study identified a 30-kDa surface protein of *C. albicans* which bound vitronectin through ligand blotting of radiolabelled vitronectin. The binding of the unknown *C. albicans* protein was also inhibited by heparin. Since Gpm1p is approximately 30-kDa in size and its binding to vitronectin is inhibited by heparin, there is a possibility that Limper et al were able to isolate Gpm1p in their study. Taken

together, Gpm1p is a surface protein of *C. albicans* which directly interacts with vitronectin by binding to its HBS. Binding of *C. albicans* to vitronectin can help the fungus to adhere to human tissues for colonization and subsequent invasion. This is the same molecular mechanism used by the bacterial pathogen *Streptococcus pneumoniae* in utilizing vitronectin to adhere to host cells and subsequent internalization. The pathogenic bacterium binds to the C-terminal HBS of vitronectin while the N-terminal integrin-binding site is free for interaction with human cells [101].

This study identifies vitronectin on the surface of both HUVEC and HaCaT cells as the ligand for Gpm1p. Pre-incubation of additional vitronectin with Gpm1p does not promote binding of the fungal protein to human cells but inhibits it, instead. Interestingly, vitronectin is present on the surface of both HUVEC and HaCaT cells as shown by flow cytometry and LSM which explains the non-binding of the vitronectin-complexed Gpm1p. This is in line with a previous study which showed that addition of vitronectin to *C. albicans* yeast cells inhibited their binding to endothelial cells because of the already-present vitronectin on the human cell surface [69]. The presence of vitronectin on the surface of endothelial cells, even after incubation in serum-free medium, was shown to be caused by the internalization and later deposition of the glycoprotein in the subendothelial matrix after exposure to vitronectin-containing serum [122,123].

Previous studies have shown that vitronectin interacts with *C. albicans* and that several vitronectin-binding proteins on the surface of the fungus exist, but none of them identified Gpm1p [67-69]. The reports showed that *C. albicans* has human integrin-like receptors which can bind vitronectin. These vitronectin-binding human integrin-like receptors on the surface of *C. albicans* were identified by flow cytometry using antibodies against the human integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. The isolated *C. albicans* vitronectin receptors were described to range from 84-130 kDa in size using biochemical characterizations. However, in this study, the antibody against $\alpha_v\beta_3$ did not recognize the 30-kDa Gpm1p in ELISA (data not shown) which indicates that it is different from the ones identified by Santoni *et al.* and Spreghini *et al.* [69,70]. The Gpm1p-vitronectin colocalization on the surface of endothelial cells and keratinocytes shown by LSM in this study further confirms that Gpm1p is a *C. albicans* surface protein which directly binds to the vitronectin on the surface of human cells. Taken together, Gpm1p is a surface protein of *C. albicans* which can aid the pathogenic fungus in human cell adhesion by binding to vitronectin. This is another mechanism by which *C. albicans* can interact with human endothelial and epithelial cells, in addition to the binding of *C. albicans* Als3 and Ssa1 to N-cadherin and E-cadherin for attachment, invasion and destruction of host tissues [34,118,124].

Gpm1p is one of the non-conventional surface proteins of *C. albicans* as it is known to be mainly present in the cytosol as an enzyme which is involved in glycolysis but has been shown to be present on the fungal surface by several

studies [37,72,125]. Other glycolytic enzymes also known to be present on the *C. albicans* cell surface are enolase (Eno1) and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) [37,72,125,126]. Currently, not enough is known as to how these glycolytic enzymes become transported to the cell surface as they lack the signal sequence for secretion. As surface proteins, Eno1 and Gapdh were described to bind the extracellular matrix proteins laminin and fibronectin, and plasminogen for tissue invasion [38,127]. Based on the results of this study, Gpm1p is the first non-conventional surface protein of *C. albicans* which is reported to bind to vitronectin.

Vitronectin is not only found in the ECM but also in serum. In this study, *C. albicans* acquires vitronectin from serum through Gpm1p. Acquisition of vitronectin from serum by pathogenic bacteria such as *Moraxella catarrhalis* [98,99], *Haemophilus influenza* [94,95] has been shown to be an immune evasion strategy since vitronectin inhibits the formation of the terminal complement complex (TCC) which lyses foreign cells in the human body. However, since *C. albicans* is not lysed by the TCC due to its thick cell wall, the function of vitronectin acquisition from serum by the fungal pathogen with regard to immune evasion is under current investigation.

4.2. *C. albicans* translation elongation factor 1-alpha as multifunctional surface protein which interacts with the complement components and neutrophils

Here, I show that translation elongation factor 1-alpha (Tef1p) of *Candida albicans* is a receptor for several complement components. Tef1p binds to the complement regulator Factor H, C4b-binding protein (C4BP), C3b degradation products C3d and C3dg, and plasminogen. The binding of Factor H by Tef1p was a confirmation of a previous study using protein array, affinity chromatography and mass spectrometry, identifying Tef1p as the fifth surface protein of *C. albicans* which bind Factor H. Among the other proteins identified are Gpm1p [61], Pra1 [62], Gpd2 [65], and Hgt1p [63].

One of the mechanisms by which microbial pathogens evade the immune system is by binding complement system regulators, such as Factor H and C4BP, mainly to degrade the opsonin C3b and block opsonization. In the continuous discovery of *C. albicans* proteins that bind Factor H, Tef1p was identified in our laboratory as one of the Complement Regulator-Acquiring Surface Proteins (CRASPs) of *C. albicans*. This study confirms the binding of *C. albicans* Tef1p to human complement system regulators by producing recombinant Tef1p. The human complement system alternative pathway regulator Factor H and the classical pathway regulator C4b-binding protein (C4BP) bind to Tef1p, which is used by *C. albicans* to avoid being recognized by the human complement system. *C. albicans* binds Factor H, FHL-1 and C4BP as an immune evasion strategy [35,62]. However, it was only recently

that specific surface proteins were identified to be Factor H and C4BP receptors. Among these surface proteins are phosphoglycerate mutase (Gpm1p) [61], pH-regulated antigen 1 (Pra1) [62], glycerol-2-phosphate dehydrogenase (Gpd2) [65], and high-affinity glucose transporter (Hgt1p) [63]. Factor H and C4BP, when bound to the aforementioned proteins, maintain their C3b degradation activity as cofactor for Factor I. Other pathogenic fungi such as *Aspergillus fumigatus* have been also shown to bind complement regulators as a strategy to evade the immune system [60]. Aside from fungi, bacterial pathogens bind human complement regulators to evade the attack of the immune system and the proteins responsible for the binding have been identified. Some of them are PspC of *Streptococcus pneumoniae*, Scl1 of *Streptococcus pyogenes*, Sbi of *Staphylococcus aureus*, and YadA of *Yersinia enterocolitica* [83,129-133].

C3b degradation products C3d and C3dg are identified in this study to be Tef1p ligands, which was confirmed by showing that *TEF1* deletion mutant strain has decreased ability to bind C3d. However, the lack of *TEF1* did not totally abolish the C3d binding capacity of the deletion strain. *C. albicans* possesses a C3d receptor on its surface and reports have described its expression, isolation and interaction with other proteins [48,49,115,134]. Previous studies were able to identify the surface C3d-binding protein by affinity chromatography and by its activity in inhibiting the rosetting of erythrocytes. The reports described the C3d receptor on *C. albicans* as a hyphae-expressed mannoprotein with a size of 62-72 kDa detected by the Complement Receptor 2 antibody. Thus, naming it as complement binding protein MP60 [48,135]. However, based on size alone, none of the studies identified the 50-52-kDa Tef1p. This indicates that Tef1p is a surface protein of *C. albicans* which binds the complement components C3d and C3dg. By binding the C3d or C3dg component of C3b, *C. albicans* inhibits the formation of C3 convertase, which subsequently inhibits C3b formation causing less opsonization of the fungus. *C. albicans* surface protein Pra1 employs this mechanism which was shown by Luo *et al.* (2011) [83]. There were also other speculations on the immune evasion functions of C3d binding by *C. albicans*. Some of them include the bridging of opsonized fungal cells and fungal cells with free binding sites for C3d, thereby producing cellular clumps which are hard to engulf by phagocytes; and shedding of the C3d receptor during infection to compete with complement receptors on phagocytes, which leads to non-detection of *C. albicans* [48]. In this study, the role of Tef1p binding of C3d has yet to be determined.

I demonstrate in this study that Factor H dose-dependently binds to C3d-bound Tef1p, forming a tripartite complex. The formation of tripartite complex consisting of a microbial protein, C3d and Factor H has been shown before for the *S. aureus* protein Sbi [83]. It was proven that Sbi-complexed C3d bound Factor H, which was active in inhibiting the complement cascade. It was therefore concluded that C3d assists in the direct binding of Factor H to Sbi.

Comparing to the results of this study, since Factor H can also bind to Tef1p alone, it can be speculated that binding of C3d/C3dg by Tef1p is an additional mechanism by which *C. albicans* can acquire more Factor H, which means stronger inhibition of the complement activation.

Tef1p binding to plasminogen is demonstrated in this study. Here, it is shown that Tef1p bound to plasminogen in a dose-dependent manner and that the interaction involves lysine residues on the Tef1p molecule (**Figure 14**). In addition, deletion of *TEF1* reduced the plasminogen binding ability of *C. albicans* but not totally abolish it (**Figure 20A**). This is consistent with the findings that *C. albicans* have several plasminogen binding proteins on its surface. As a plasminogen receptor on the surface of *C. albicans*, Tef1p was previously identified by Crowe *et al.* (2003) by using affinity chromatography [37,38]. Tef1p is one of several plasminogen binding surface proteins of *C. albicans*, together with catalase, thioredoxin peroxidase, phosphoglycerate mutase (Gpm1p), alcohol dehydrogenase (Adh1), glyceraldehyde-3-phosphate dehydrogenase (Gapdh), phosphoglycerate kinase (Pgk1), fructose bisphosphate aldolase (Fba1), Pra1, Gpd2 and Eno1 [37,38,61,62,65].

Aside from the normal degradation activity of the natural substrates fibrinogen and vitronectin, the novel function of plasminogen binding by Tef1p is shown in this work to be the degradation of the C3b opsonin for immune evasion. Tef1p-bound plasminogen is accessible to the activator tPA which transforms plasminogen to the protease plasmin. The Tef1p-bound plasmin can then degrade the opsonin C3b into inactive fragments, thereby rendering *C. albicans* unrecognizable by phagocytes.

Activation of the plasminogen bound on the surface of *C. albicans* leads to the degradation of the opsonin C3b, which leads to immune evasion by the fungal pathogen. It was indicated that the reason for binding of plasminogen by *C. albicans* is to degrade the extracellular matrix proteins on human tissues, which is the natural substrate of plasmin, to facilitate the invasion of the fungus. Eventhough it has been shown that plasmin-bound *Candida* cells can induce fibrinolysis and increase ability to cross an in vitro blood-brain barrier system, *C. albicans* can still destroy the endothelial matrix, and penetrate and damage endothelial cells in the absence of plasminogen [37]. The mechanism of plasminogen binding by *C. albicans* for C3b degradation is new from this study. Previous reports have shown that it is a common strategy used by bacteria to evade phagocytosis. *Haemophilus influenza* PE, *Bacillus anthracis* α -enolase and EF-tu, and *S. aureus* Sbi and Efb have all been shown to bind plasminogen, which when activated to the protease plasmin, degrades the opsonin C3b [5,84,110].

Tef1p localization in *C. albicans* cytoplasm and expression on the cell surface was investigated using the generated antiserum against Tef1p in Western blotting (**Figure 17B**). Tef1p expression on both the yeast and hyphal cells of *C. albicans* was also confirmed using flow cytometry (**Figure 17C**) and LSM

(Figure 17D). Tef1p presence on the surface of *C. albicans* was previously shown by Crowe *et al.* [37] and Urban *et al.* [79]. However, expression of Tef1p on hyphal cells is shown here to be stronger than on yeast cells. These results are in line with the gene expression studies done by Singh *et al.* [136] and Zakikhany *et al.* [81] which showed that *TEF1* gene is upregulated in pseudohyphae and hyphae forms during incubation with macrophages and epithelial cells.

Deletion of *TEF1* does not affect the growth of the fungus even in different environmental conditions. The previous speculation that *TEF1* is an essential gene [87] has been disproven based on the results of this study. Here it is shown that even in the absence of *TEF1*, *C. albicans* can still grow like the wild type **(Figure 19A and 19B)**. *TEF1* deletion strain can also resist cell wall perturbing agents; and osmotic and oxidative stress. This implies that *TEF1* is not essential in basic growth and metabolic functions of *C. albicans*. However, the protein Tef1p could still be essential since the protein is encoded by two genes, *TEF1* and *TEF2* [80]. Since only *TEF1* gene was deleted in this study, the possibility that *TEF2* encodes Tef1p and performs its essential role cannot be discounted. This is based on the fact that both *TEF1* and *TEF2* are translated equally and that *C. albicans* does not preferentially use one gene over the other [137]. This is also the case for the yeast *Saccharomyces cerevisiae* where deletion of *TEF1* did not affect its growth. This was also attributed to the presence of *TEF2* which encodes the same translation elongation factor EF-1 α [138].

However, deletion of *TEF1* makes *C. albicans* sensitive to the antibiotic hygromycin B **(Figure 19F)**. Hygromycin B is an aminoglycoside that affects translational elongation by binding distinct sites at the ribosome in relative proximity to the ribosomal decoding center, thereby reducing translational fidelity [139]. The sensitivity of *C. albicans* *TEF1* deletion strain to hygromycin B indicates that the encoded Tef1p plays a critical role in counteracting the effects of the antibiotic. This was demonstrated by De Backer [140] wherein overexpression of the Tef1p caused by depletion of mRNA 59-guanylyltransferase gene (CGT1) in *C. albicans* led to the reduced sensitivity to hygromycin B. The result of this study is also in line with what was shown for *S. cerevisiae* wherein mutant forms of *TEF2*, which also encodes for the same protein as *TEF1* [137] made the yeast sensitive to hygromycin B [141]. The possibility that *C. albicans* *TEF2* could take over the production of Tef1p in the absence of *TEF1* for counteracting the effects of hygromycin B cannot be ignored. But based on this work, The Tef1p produced by *TEF2* alone is not sufficient to maintain translational fidelity in the presence of hygromycin B.

Tef1p binding to neutrophils is demonstrated in this study. In addition to Pra1, Tef1p is identified as a *C. albicans* protein surface component which interacts with neutrophils. It is known that receptors on neutrophils recognize different components of the fungal pathogen's surface. Dectin-1 recognizes the β 1-3 glucan while Toll-like receptors collaborate with Dectin-1 in recognizing the

fungus using surface mannans. Another receptor on the surface of neutrophils is complement receptor 3 (CR3) which recognizes opsonized *C. albicans* cell. Opsonins C3d and iC3b opsonize the fungal pathogen and is recognized by CR2 and CR3, respectively. However, it has been shown that non-opsonized *C. albicans* cells are also recognized by neutrophils. It was discovered that Pra1 is directly recognized by CR3. However, since it is shown in this work that the lack of *TEF1* decreases the ROS released by neutrophils, it suggests that Tef1p is specifically recognized by neutrophils and has a role in activating the myeloperoxidase-H₂O₂-halide system. The role of Tef1p in the production of ROS by neutrophils is highly speculative. It could be that Tef1p is recognized by neutrophils using another receptor since addition of antibodies against CD11b and CD18 (components of the integrin) did not block the binding of Tef1 to neutrophils (data not shown). But since there is a possibility that Tef1p is the C3d receptor with similarity to the CR3 sequence, it could be that the effect of the CR3 antibodies was counteracted. Taken together, the binding of *C. albicans* Tef1p could be an indication that the protein is recognized by the neutrophils and triggers a signaling cascade which induces the production of ROS.

CONCLUSION

In summary, this study characterizes two *C. albicans* surface proteins and their interaction with the human host. The proteins Gpm1p and Tef1p contribute to the adhesion to human tissue and evasion of the innate immune system, respectively. On one hand, Gpm1p binds vitronectin for adhesion to human tissues. It is described how Gpm1p specifically interacts with vitronectin and localize with the native vitronectin on the surface of both endothelial cells and keratinocytes. The *C. albicans* strain lacking the *GPM1* gene has decreased ability to be associated (adhered and/or endocytosed) with endothelial cells. On the other hand, Tef1p interacts with several components of the innate immune system for immune evasion. Tef1p binds the complement regulatory proteins Factor H and C4BP. Tef1p also binds the C3b fragments C3d and C3dg. A tripartite complex is shown to form between Tef1p, C3d and Factor H. In addition, Tef1p binding to plasminogen shows that the opsonin C3b can be degraded making it inactive for phagocyte recognition. Moreover, *C. albicans* Tef1p has a role in the interaction between the fungus and human neutrophils as evidenced by the inability of neutrophils to produce ROS when challenged with *C. albicans* lacking the *TEF1* gene. It is interesting to note that both Gpm1p and Tef1p are non-canonical surface proteins of *C. albicans* due to the lack of secretion signal in their genetic sequences, which makes them moonlighting proteins. Knowledge of the mechanisms on how these proteins translocate to the surface of *C. albicans* is still nil and needs further investigation.

The findings of this study contribute to the further understanding on how *C. albicans* interacts with the human host using surface proteins. Application of the results presented here can be extended to clinical settings and discover how, for example, Gpm1p can be inhibited to bind to vitronectin on the surfaces of implants, such as catheters, in hospital patients. Another possibility is to find ways to disarm *C. albicans* in evading the immune attack by inhibiting the surface Tef1p.

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DECLARATION OF HONOUR

I hereby declare on my honour that I am familiar with the relevant course of examination for doctoral candidates of the Faculty of Biology and Pharmacy of the Friedrich-Schiller-Universität Jena.

I also declare that I personally composed and wrote the dissertation and that I acknowledged all additional assistance, personal communications, and sources according to the rules of academic work within this dissertation.

I declare that assistance provided by specific individuals during the study and writing of this dissertation has been indicated in full.

I declare that I did not enlist any assistance of a doctoral consultant and that no third parties have received either direct or indirect monetary benefits from me for work connected to this submitted dissertation.

I declare that this dissertation has not been submitted as an examination paper for a state or other scientific examination.

I also declare that I did not submit the same, a substantially similar, or a different paper to another postsecondary school.

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The roles of *Candida albicans* surface proteins in the immune evasion and tissue invasion of the human host

Department of Infection Biology, Leibniz Institute for Natural Product Research and Infection Biology (Hans Knöll Institute), Jena, Germany (*Ph.D. dissertation*)

Heterologous expression of endoglucanase genes from endosymbiotic protists of the lower termite *Reticulitermes speratus* in *Aspergillus oryzae*

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Antimicrobial activity of bacteriocin produced by lactic acid bacteria isolated from various Philippine fermented food products

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SCHOLARSHIPS RECEIVED

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LIST OF SCIENTIFIC PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

Otagiri, M., **C.M. Lopez**, K. Kitamoto, M. Arioka, T. Kudo and S. Moriya. 2012. Heterologous expression and characterization of a glycoside hydrolase family 45 endo-beta-1,4-glucanase from a symbiotic protist of the lower termite, *Reticulitermes speratus*. *Appl. Biochem. Biotechnol.* Accepted.

Todaka, N., **C.M. Lopez**, T. Inoue, K. Saita, J. Maruyama, M. Arioka, K. Kitamoto, T. Kudo and S. Moriya. 2010. Heterologous expression and characterization of an endoglucanase from a symbiotic protist of the lower termite, *Reticulitermes speratus*. *Appl. Biochem. Biotechnol.* 160:1168-1178.

Lopez, C.M., S. Nitisinprasert, P. Wanchaitanawong and N. Poovarodom. 2003. Antimicrobial Activity of Medicinal Plant Extracts against Foodborne Spoilage and Pathogenic Microorganisms. *Kasetsart J. (Nat. Sci.)*. 37(4): 460-467.

ORAL PRESENTATIONS

Title: The surface-associated translation elongation factor 1 of *Candida albicans* mediates its interaction with the innate immune system
(**Lopez, C.M.**, P. Miramon, S. Duggan, O. Kurzai, B. Huber and P.F. Zipfel)
Event: 18th Congress of International Society for Human and Animal Mycology
Date: June 11-15, 2012
Location: Berlin, Germany

Title: The translation elongation factor 1 of *Candida albicans* is a surface protein which uses complement regulatory proteins for immune evasion
Event: Statusworkshop der DGHM Fachgruppe Eukaryontische Krankheitserreger
Date: February 17-18, 2012
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Grant: Travel grant

Title: Characterization of *Candida albicans* surface translation elongation factor 1 and its role in the interaction with human host
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Event: 2nd JSMC International Student Conference on Microbial Communication (MiCom 2011)
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Scientific Publications and Presentations

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POSTER PRESENTATIONS

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Title: Surface phosphoglycerate mutase mediates adherence of *Candida albicans* to human endothelial cells (**Lopez, C.M.** and P.F. Zipfel)
Event: ILRS Symposium 2011
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Title: Phosphoglycerate mutase of *Candida albicans* is essential in hyphae formation and subsequent adherence to human endothelial cells (**Lopez, C.M.**, S. Poltermann, M. von der Heide and P.F. Zipfel)
Event: European Student Conference on Microbial Communication 2010 (MiCom)
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Scientific Publications and Presentations

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Title: Surface phosphoglycerate mutase of *Candida albicans* interacts with vitronectin and binds to both endothelial and epithelial human cells
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Event: JSMC Symposium 2008
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THESIS STATEMENTS

of the dissertation of Crisanto M. Lopez, M.Sc.

The opportunistic fungus *Candida albicans* possesses virulence factors which are used to invade human tissues and evade the immune system. The ability to bind to human tissues for subsequent colonization and invasion, and to utilize human immune regulatory proteins for its own protection is achieved by *C. albicans* by expressing specific proteins on its cell surface. It was therefore the aim of this work to identify cell surface-associated proteins of *C. albicans* and elucidate the roles that they play in the virulence of the opportunistic fungus. The main findings of the study are:

1. *C. albicans* uses its cell surface protein phosphoglycerate mutase (Gpm1p) to adhere to human umbilical vein endothelial cells (HUVEC) and keratinocytes (HaCaT). Deletion of the *GPM1* gene which expresses Gpm1p decreases adherence of *C. albicans* to human cells.
2. Gpm1p binds to vitronectin expressed by HUVEC and HaCaT. Recombinant Gpm1p colocalizes to the vitronectin expressed on the surface of HUVEC and HaCaT. Moreover, soluble vitronectin from human serum is bound by *C. albicans* through Gpm1p.
3. Gpm1p interacts with vitronectin by binding to the heparin binding site on the vitronectin molecule.
4. *C. albicans* expresses translation elongation factor 1-alpha (Tef1p) on the cell surface of both yeast and hyphal forms.
5. *C. albicans* Tef1p binds complement regulatory proteins Factor H and C4BP; and C3 fragments C3b, C3d, C3dg and iC3b. Binding to these proteins could help *C. albicans* in the avoidance of being recognized by phagocytes.
6. *C. albicans* Tef1p binds plasminogen which, when activated to plasmin by tissue-type plasminogen activator, maintains its degradation activity on synthetic substrate S-2251 and extracellular matrix proteins fibrinogen and vitronectin.
7. *C. albicans* uses Tef1p to bind to plasminogen to degrade the opsonin C3b. Homozygous deletion of *TEF1* (orf19.1435) decreases the

binding ability of *C. albicans* to plasminogen which could lead to less C3b degradation capability of *C. albicans*.

8. When *TEF1* of *C. albicans* is deleted, the mutant shows regular growth compared to the wildtype in normal fungal cultivation conditions. This could be accounted for the presence of the second *C. albicans* gene *TEF2* (orf19.382) which encodes for an identical Tef1p.
9. *C. albicans TEF1* is dispensable for the resistance to osmotic and oxidative stresses, and cell wall perturbing agents. In contrast, *TEF1* deletion leads to increased sensitivity to hygromycin.
10. *C. albicans* uses Tef1p to interact with neutrophils. Neutrophils produce less reactive oxygen species when *TEF1* is lacking in *C. albicans*.
11. Gpm1p and Tef1p are important virulence factors of *C. albicans* in the interaction with the human host.

THESEN

zur Dissertation von Crisanto M. Lopez, M.Sc.

Der opportunistische Pilz *Candida albicans* besitzt Virulenzfaktoren, die zur Invasion des menschlichen Gewebes und zur Evasion des Immunsystems verwendet werden. Die Fähigkeit an humane Gewebe zur nachfolgenden Kolonisierung und Invasion zu binden, sowie die Verwendung immunregulatorischer Proteine des Menschen zum Schutz der eigenen Zellen erreicht *C. albicans* durch die Expression spezieller Proteine auf seiner Oberfläche. Das Ziel der vorliegenden Dissertation war die Identifikation Zelloberflächen-assoziiierter Proteine und der Aufklärung ihrer Rolle im Virulenz-regulatorischen Netzwerk von *C. albicans*. Die bedeutendsten Ergebnisse dieser Arbeit sind:

1. *C. albicans* verwendet das Zelloberflächen-Protein Phosphoglyceratmutase (Gpm1p) um an Humane Umbilikalvene-Endothelzellen (HUVEC) und Keratinozyten (HaCaT) zu binden. Die Deletion von GPM1, welches das für Gpm1p-kodierende Gen ist, führt zu einer Abnahme der Adhäsion von *C. albicans* an humane Zellen.
2. Gpm1p bindet an Vitronectin, welches von HUVEC und HaCaT exprimiert wird. Rekombinantes Gpm1p ist in der Lage, mit dem von HUVEC und HaCaT exprimierten Vitronectin zu kolokalisieren. Weiterhin ist *C. albicans* durch Expression von Gpm1p in der Lage, in humanem Serum gelöstes Vitronectin zu komplexieren.
3. Gpm1p interagiert mit Vitronectin und bindet es über die Heparin-Bindestelle im Vitronectin-Molekül.
4. *C. albicans* exprimiert den Translationselongationsfaktor 1- α (Tef1p) auf der Zelloberfläche sowohl von Hefezellen als auch auf Hyphen.
5. Tef1p von *C. albicans* bindet die Komplement-regulatorischen Proteine Faktor H und C4BP, sowie C3 und dessen Fragmente C3b, C3d, C3dg und iC3b. Die Bindung an diese Faktoren kann *C. albicans* dabei helfen, die Erkennung durch Phagozyten zu verhindern.
6. Plasminogen wird von *C. albicans* Tef1p gebunden und behält, nach dessen Aktivierung zu Plasmin durch den Gewebsspezifischen Plasminogenaktivator, seine Fähigkeit zur Degradation des

synthetischen Substrats S-2251 und den Extrazellulären Matrixproteinen Fibrinogen und Vitronectin bei.

7. *C. albicans* verwendet Tef1p zur Bindung von Plasminogen, um das Opsonin C3b zu degradieren. Die homozygote Deletion von *TEF1* (orf19.1435) führt zu einer Abnahme der Bindefähigkeit von *C. albicans* an Plasminogen, was zu einer verringerten Fähigkeit der C3b-Degradierung von *C. albicans* führen könnte.
8. Nach einer Deletion von *TEF1* in *C. albicans* zeigt die entsprechende Mutante ein zum Wildtyp vergleichbares Wachstum unter Standard-Kulturbedingungen. Dies kann zurückzuführen sein auf die Anwesenheit des Gens *TEF2* (orf19.382), welches in *C. albicans* für die Expression eines identischen Tef1p kodiert.
9. *C. albicans tef1* trägt nicht bei zur Resistenz gegenüber osmotischem und oxidativem Stress, sowie gegenüber Zellwand-störenden Agenzien bei. Im Gegensatz führt die Deletion von *TEF1* zu einer erhöhten Sensitivität gegenüber dem Antimykotikum Hygromycin.
10. *C. albicans* verwendet Tef1p um mit Neutrophilen zu interagieren. Diese zeigen eine geringere Bildung von Reaktiven Sauerstoffspezies wenn Tef1p in *C. albicans* fehlt.
11. Die gezeigten Ergebnisse lassen den Schluss zu, dass die Proteine Gpm1p und Tef1p von *C. albicans* wichtige Virulenzfaktoren für die Interaktion mit den Menschen als Wirt darstellen.